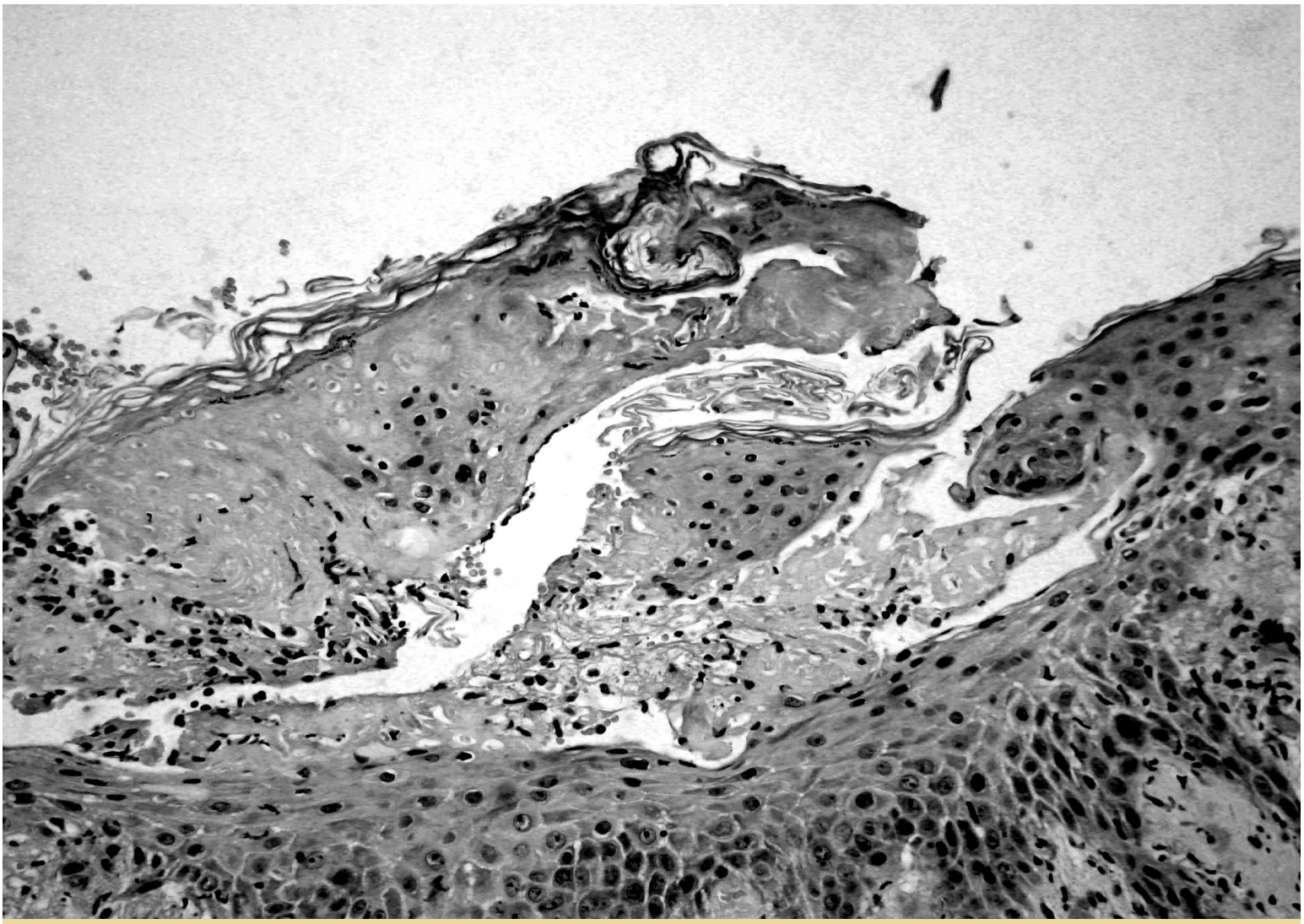

Maria Margarida Martins Gonçalo

CUTANEOUS ADVERSE DRUG REACTIONS

Contributions to understand pathophysiologic mechanisms involved in delayed reactions

Tese de Doutoramento em Ciências da Saúde, Ramo de Medicina, Especialidade de Medicina Interna (Dermatologia e Venereologia)
orientada por Américo Manuel Costa Figueiredo e Maria Celeste Fernandes Lopes
e apresentada à Faculdade de Medicina da Universidade de Coimbra

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· U C ·

Universidade de Coimbra

To Hugo

To my Parents

To my Family

To my Teachers and Masters

To my Colleagues and Friends

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ABSTRACT

Delayed immune mediated cutaneous adverse drug reactions (CADR) have several challenging aspects in their pathophysiology. Similarities with pathophysiologic mechanisms involved in allergic contact dermatitis (ACD) were a rational for conducting most of this investigation.

In ACD patch tests are positive and reproduce the eczema. In delayed CADR, patch test reactivity depends on the culprit drug and the pattern of CADR. Histopathology of patch tests in 18 patients (7M/11F, mean age 60.5y) showed a perivascular dermal infiltrate with epidermal spongiosis and exocytosis, mainly of neutrophils or lymphocytes, depending on the type of CADR. In 4/5 biopsies from acute generalized exanthematous pustulosis, neutrophil subcorneal pustules simulated the acute eruption. In maculopapular exanthema (6), drug reaction with eosinophilia and systemic symptoms (DRESS) (5) and Stevens-Johnson Syndrome/Toxic epidermal necrolysis (SJS/TEN) (2) a lymphomononuclar infiltrate and exocytosis predominated, in the latter cases with vesicles and extensive keratinocyte necrosis. The intensity of epidermal aggression and inflammatory infiltrate in the patch test increased from maculopapular exanthema to DRESS and SJS/TEN. Similarities between the macro/microscopic features of the patch test and the acute drug eruption reinforce the specificity of patch testing and its usefulness in studying CADR pathophysiologic mechanisms.

In 15 patients (9M/6F, mean age 53.3y) with DRESS, mainly from allopurinol (8) or anticonvulsants (5), histopathology of the exanthema showed, in variable intensity and proportions, a lymphocyte and/or eosinophil dermal infiltrate, lymphocyte exocytosis, spongiosis, scattered necrotic or vacuolated keratinocytes, suggesting a cytotoxic effect from infiltrating cells. No specific aspect allowed the diagnosis of DRESS or its relation with the culprit drug or viral reactivation, as there were overlapping aspects between DRESS, SJS/TEN (bullous forms with epidermal cytolysis) and maculopapular exanthema. We

found, nevertheless, a significant positive correlation between the intensity of lymphocyte infiltration and the severity of hepatic cytolysis ($r=0.51$, $p<0.05$). If this finding is confirmed in larger studies, together with the previously described relation between intensity of liver cytolysis and epidermal necrosis, histopathology can be an additional prognostic marker in DRESS.

HLA-B*58:01 is considered a risk factor for severe CADR from allopurinol, mainly in Han Chinese. HLA genotyping in 25 Caucasian patients (11M/14F, mean age 67.4y) with DRESS (19) or SJS/TEN (6) from allopurinol and in 23 allopurinol exposed and tolerant controls, showed HLA-B*58:01 in 16/25 patients (64%) (12 DRESS, 4 SJS/TEN), in 1/ 23 controls (4.3%) and in 1.96% of the normal population. Compared with tolerant individuals, this represents an OR=37.71 (95% CI: 4.13-343.87) for DRESS and OR=44.0 (95% CI: 3.18-608.19) for SJS/TEN. Considering the normal population it represents an OR=85.36 (95% CI: 32.52-224.04) for DRESS and OR=99.59 (95% CI: 17.91-553.72) for SJS/TEN. In Europeans, a similar risk was shown only for SJS/TEN, and this study extends it also to DRESS. Although very significant, this risk is inferior to the Chinese where almost all patients with severe reactions from allopurinol carry this allele. The combination of a low prevalence of HLA-B*58:01 in the Portuguese population and lower associated risk, question the cost-benefit of systematic pretesting before using allopurinol.

To fill a gap in the knowledge of the initial sensitizing phases of CADR, we evaluated the *in vitro* effect of systemic drugs on a human dendritic-like cell line (THP-1), which suffers maturation and activation upon exposure to contact sensitizers. Cells exposed to carbamazepine, sodium valproate, allopurinol and its active metabolite, oxypurinol, amoxicillin and ampicillin, showed, by WB, activation of p38 MAPKinase and, by RT-PCR, upregulation of genes coding for the cell detoxifying enzyme hemeoxygenase 1 (*HMOX-1*) and for IL-8/CXCL8. Genes for dendritic cell maturation makers (*CD83, CD40*) and pro-inflammatory cytokines (*IL12B, CXCL10*) were also upregulated by some drugs. THP-1 cell activation by allopurinol and oxypurinol was very significant and similar to the strong sensitizer, DNFB. Unexpectedly, sodium valproate activated more pathways than carbamazepine. Although somehow

divergent, results suggest that, like contact sensitizers, drugs induce an innate immune response in human cells that may be a significant “danger signal” to awaken the adaptive immune response in CADR.

This study, with several limitations, brought some additional contributions to the understanding of pathophysiologic aspects of CADR, namely the existence of a drug-induced innate xenoinflammation that may favour antigen presentation. It defined HLA-B*58:01 as a risk factor for severe CADR from allopurinol, including DRESS, in our population. Histopathology of DRESS exanthema showed correlation with severity markers and histopathology of positive patch tests, with significant similarities to the acute eruption, was shown to be interesting to study effector mechanisms in CADR.

SUMÁRIO

As reações cutâneas adversas a medicamentos (CADR) não imediatas apresentam várias lacunas no conhecimento dos mecanismos imunopatológicos que lhe estão subjacentes. Tivemos por base as semelhanças fisiopatológicas com a dermatite de contacto alérgica (ACD) para melhor entender os mecanismos das CADR.

Na ACD os testes epicutâneos reproduzem o eczema. Nas CADR a reatividade depende do medicamento e do padrão de reação. Neste estudo, a análise histopatológica de 18 testes epicutâneos positivos a medicamentos mostrou um infiltrado predominantemente linfocitário ou neutrofilico com variável agressão epidérmica, semelhante ao exantema da CADR. Em 4/5 biopsias de pustulose exantemática aguda generalizada (AGEP) existiam pústulas subcórneas. No exantema maculopapular (6), reação medicamentosa com eosinofilia e sintomas sistémicos (DRESS) (5) e síndrome de Stevens-Johnson/necrólise epidérmica tóxica (SJS/TEN) (2) predominava o infiltrado linfomononuclear dermo-epidérmico com vacuolização, necrose ceratinoticitária e formação de vesículas, mostrando uma intensidade crescente nas formas mais severas de CADR e semelhança macro e microscópica com as lesões agudas da CADR. Para além de reforçar a especificidade, esta semelhança histopatológica permite estudar a resposta efectora também no teste epicutâneo.

Em 15 pacientes com DRESS (9 M/6 F, idade média 53.3A) induzido sobretudo pelo alopurinol (8) ou anticonvulsivantes (5), o estudo histopatológico do exantema revelou exocitose linfocitária, espongiose, vacuolização ou necrose ceratinocitária e infiltrado dérmico com linfócitos e/ou eosinófilos, em padrões do tipo do eritema polimorfo, dermatite espongiótica, reação liquenóide ou pseudolinfoma, sem qualquer aspecto específico de DRESS ou relacionado com o medicamento causal ou infecção viral concomitante e com formas de sobreposição histológica entre DRESS, SJS/TEN e exantema maculopapular. A correlação positiva e significativa entre a intensidade do infiltrado linfocitário e

a gravidade da citólise hepática ($r=0.51$, $p<0.05$) a confirmar-se em estudos mais alargados, tal como a correlação entre a intensidade da necrose epidérmica e citólise hepática relatada noutro estudo, apontam para o interesse da histopatologia como marcador de prognóstico no DRESS.

O HLA-B*58:01 é um factor de risco para CADR graves ao alopurinol, sobretudo em Chineses Han. A tipificação HLA em 25 pacientes Caucasianos (11M/14F, idade média 67.4 A) com DRESS (19) ou SJS/TEN (6) e em 23 controlos expostos ao alopurinol sem reação adversa, revelou a presença de HLA-B*58:01 em 16/25 pacientes (12 DRESS, 4 SJS/TEN), em 1/23 controlos e em 1.96% da população geral. Assim, o HLA-B*58:01 representa um risco significativo para DRESS (OR=37.71; 95% CI 4.13-343.87 ou OR=85.36; 95% CI 32.52-224.04, respectivamente quando comparado com os indivíduos tolerantes ao fármaco ou a população normal) e para SJS/TEN (respectivamente, OR=44.0; 95% CI 3.18-608.19 ou OR=99.59; 95% CI 17.91-553.72). Em Europeus tinha sido documentado um risco semelhante no SJS/TEN e, com este estudo, o risco foi alargado também ao DRESS. Apesar de significativo, o risco é muito inferior ao dos Chineses Han em que quase todos os pacientes com CADR graves ao alopurinol são HLA-B*58:01 positivos. O risco mais baixo associado à prevalência inferior do HLA-B*58:01 na população portuguesa, questiona o custo-benefício de tipificação sistemática do HLA antes de iniciar alopurinol.

Avaliámos o efeito *in vitro* de fármacos sistémicos em células THP-1 que, tal como as células dendríticas, sofrem maturação e ativação após exposição a sensibilizantes de contacto. Usando WB e RT-PCR, nas células expostas à carbamazepina, valproato de sódio, alopurinol, oxipurinol, amoxicilina e ampicilina, verificámos ativação da p38 MAPKinase e aumento na transcrição de genes que codificam hemeoxigenase-1 (*HMOX-1*), enzima de detoxificação celular, e *IL-8/CXCL8*, quimiocina da resposta inata. De forma mais irregular, foram ativados genes de marcadores pró-inflamatórios ou de maturação da célula dendrítica (*CD83, CD40, IL12B, CXCL10*). O alopurinol e oxipurinol induziram uma reação intensa e semelhante ao potente sensibilizante, DNFB. Inesperadamente, a carbamazepina foi muito pouco reativa neste modelo em contraste com o valproato de sódio, causa mais rara de CADR. Apesar de

alguma divergência, estes dados sugerem que, tal como os sensibilizantes de contato, os fármacos sistémicos podem induzir uma resposta inata de alarme em células da pele e de outros órgãos, ativando o sistema imune adaptativo e facilitando a CADR.

Estes estudos, apesar de algumas limitações, esclareceram aspectos do mecanismo fisiopatológico das CADR, no que concerne a factores de risco, à capacidade de fármacos induzirem uma reação inata facilitadora da CADR, à possibilidade de a histopatologia do DRESS ser um marcador de prognóstico e de os testes epicutâneos serem utilizados também no estudo de mecanismos efectores envolvidos nas CADR.

LIST OF ABBREVIATIONS

ACD	allergic contact dermatitis
ADCC	antibody-dependent cellular cytotoxicity
AGEP	acute generalized exanthematous pustulosis
AIDS	acquired immunodeficiency syndrome
ATP	adenosine-triphosphate
BSA	body surface area
CADR	cutaneous adverse drug reaction
CCL or CXCL	CC or CXC chemokine ligand
CCL2/MCP-1	monocyte chemotactic protein-1
CCL5/RANTES	regulation and activated normal T cell expressed and secreted
CCL17/TARC	thymus and activation-regulated chemokine
CCL22/MDC	macrophage-derived chemokine
CCL27/CTACK	cutaneous T-cell attracting chemokine
CD	cluster of differentiation
CLA	cutaneous leukocyte antigen
CMV	cytomegalovirus
CTL	cytotoxic T lymphocytes
CXCR..n	chemokine receptor ..n
CYP	cytochrome P 450 enzymes
DAMP	death/damage-associated molecular patterns
DC	dendritic cell
DIHS	drug induced hypersensitivity syndrome
DNA	deoxyribonucleic acid
DRESS	drug reaction with eosinophilia and systemic symptoms
EBV	Ebstein-Barr virus
ELISA	enzyme linked immunosorbent assay
ELISpot	enzyme-linked immunospot
FasL	Fas-ligand
GM-CSF	granulocyte-monocyte colony stimulating factor
GWAS	genome-wide association studies
h-CLAT	human-cell line activation test
HAART	highly active antiretroviral treatment
HAF	hyaluronic acid fragments
HHV-6	Human herpes virus type 6
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMGB-1	high mobility group box 1 protein
ICAM-1	intercellular adhesion molecule-1
IFN-γ	γ-interferon
Ig	immunoglobulin
IL12B	subunit B of IL-12 (IL-12p40)
IL-n	interleukin – n

iNOS	inducible nitric oxide synthase
CXCL10	Interferon induced protein of 10 KDa/CXCL10
IRIS	immune reconstitution inflammatory syndrome
IVIG	intravenous immunoglobulins
Keap1-Nrf2-ARE	Kelch-like ECH-associated protein/Nuclear erythroid factor-related factor-2/antioxidant response element
LFA-1	lymphocyte function antigen-1
LPS	lipopolysaccharide
LTT/LST	lymphocyte transformation test; lymphocyte activation test
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MMP-9	metalloproteinase 9
NF-κB	nuclear factor of transcription κB
NK	natural killer
NKT	natural killer T cells
NLRP3	nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3
NO	nitric oxide
NOD	nucleotide-binding domain
NSAID	nonsteroidal anti-inflammatory drugs
PAF	platelet activation factor
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
RAST	radioallergosorbent test
ROS	reactive oxygen species
SCORTEN	severity-of-illness score for toxic epidermal necrolysis
SDRIFE	symmetrical drug-related intertriginous and flexural exanthema
SJS	Stevens-Johnson syndrome
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
Tc	cytotoxic T cell
TCR	T-cell receptor
TEN	toxic epidermal necrolysis
TGF	transforming growth factor
Th	helper T cell
THP-1	human monocyte derived T cell line
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cells
TWEAK	TNF-related weak apoptosis inducer
UV/UVA	ultraviolet/ultraviolet A
WHO	World Health Organization

A.
GENERAL INTRODUCTION

A. GENERAL INTRODUCTION

Cutaneous adverse drug reactions (CADR) are a frequent and challenging clinical issue in our daily practice in Dermatology. They involve complex and incompletely understood pathophysiologic mechanisms and manifest under different clinical patterns varying from mild to severe life-threatening CADR.

The number of drug adverse events is high and is, very probably, increasing. A higher life expectancy and a wider access to healthcare associated with more intensive treatments with multiple drugs, some recently released into the market, may explain this high frequency.

For incompletely understood reasons, the skin is frequently involved in the expression of these adverse events. Precise epidemiologic data are missing, in a certain way due to the difficulty in having a definitive diagnosis of CADR. Except from a Danish study reporting drug eruptions only in 0.33% of in-patients (Borch et al. 2006), CADR are reported to affect 2% to 6% of in-patients (Roujeau 2005),(Demoly et al. 2007),(Mockenhaupt 2012). Moreover, in our experience, CADR are a frequent cause of urgent dermatological observations (Fernandes et al. 2000) and, due to the severity of some patterns, they are a frequent cause for admission to our ward.

Apart from the high number of CADR, new clinical patterns of the cutaneous expression of adverse drug events are regularly described. In addition to the classical drug reactions, like urticaria, angioedema, anaphylaxis, generalized maculopapular exanthema, Stevens-Johnson Syndrome (SJS), toxic epidermal necrolysis (TEN) and fixed drug eruption, acute generalized exanthematous pustulosis (AGEP) and drug reaction with eosinophilia and systemic symptoms (DRESS) have been characterized in the last decades. With the advent of new targeted therapies, the skin is often secondarily involved by side–effects. They range from papulo-pustular eruptions, ingrown nails and hair problems during therapy with epidermal growth factor-receptor (EGF-R) inhibitors (Santiago et

al. 2011) to the apparently unrelated association of photosensitivity, keratoderma and newly developing keratoacanthoma ("rasopathies") during therapy with vemurafenib, the V600E BRAF mutation inhibitor for the therapy of metastatic melanoma (Rinderknecht et al. 2013).

CADR are also a challenge to our diagnostic capacity. Although there are some very typical patterns (AGEP, SJS/TEN and fixed drug eruption), most often CADR present as maculopapular exanthema or urticaria that may have other aetiology. Moreover, CADR can mimic skin diseases which are not usually drug-induced, like lichen plannus, psoriasis, lupus erythematosus or pemphigus vulgaris (Roujeau 2005),(Pirmohamed et al. 2011). The time course of the different CADR is also very variable. They occur within minutes, hours, days, weeks or even months after drug administration and may last a few hours to weeks, months or years. Moreover, virtually any drug can induce a CADR, each drug can induce several clinical patterns of CADR and there is no universal test to confirm drug hypersensitivity.

With this wide spectrum of clinical presentations, in the 21st century, we can consider CADR as the great skin imitator, replacing the position occupied by syphilis in the previous centuries.

Most CADR are mild and respond easily to drug suspension and symptomatic therapy. But, apart from the skin, systemic organs can be additionally involved, as in DRESS or TEN, and it is the responsibility of the Dermatologist to recognize cutaneous alarming signs as, for the moment, biomarkers for severe CADR are not yet widely available.

Therapy of severe life-threatening CADR, like SJS/TEN or DRESS, can be challenging for the Dermatologist and collaboration with other specialities is welcome. New discoveries on the pathophysiology are expected to be translated into more effective therapy, namely against the mediators of keratinocyte apoptosis in SJS/TEN or against virus that may concur with drugs to induce severe cases of DRESS.

Another challenging aspect of CADR is the identification and confirmation of the culprit drug. In immune mediated CADR new diagnostic tests, or improvement of the existent ones, can benefit from a better knowledge of pathophysiologic mechanisms, but they are far from being standardized and widely available. Therefore, in the study of delayed or nonimmediate immune CADR, we have been using patch testing, mainly indicated for the study of allergic contact dermatitis (ACD). Both delayed CADR from systemic drugs and ACD from contact sensitizers are caused by simple chemicals and they also share immune mechanisms (type IV hypersensitivity reaction), some clinical presentations (generalized maculopapular reactions) and diagnostic methods (patch testing, lymphocyte stimulation tests) (Peiser et al. 2012). In ACD, the eczematous reaction with dermal and epidermal T cell infiltration is reproduced in epicutaneous patch tests, and this also occurs in some delayed CADR. Moreover, systemic exposure to drugs or chemicals to which individuals were primarily sensitized through the skin, can induce systemic contact dermatitis presenting as maculopapular exanthema, "baboon syndrome" or SDRIFE (symmetrical drug-related intertriginous and flexural exanthema), which have many similarities with delayed CADR (Hausermann et al. 2004),(Winnicki and Shear 2011),(Veien 2011). Therefore, and considering these similarities, ACD and patch testing have been an important source of data for the understanding of pathophysiologic mechanisms involved in CADR from systemic drugs and, particularly, for the present study.

Histopathology of positive patch tests with drugs in different patterns of CADR and their correlation with the acute eruption may further strengthen the relation between ACD and CADR and reinforce the usefulness of these tests in studying pathophysiologic mechanisms involved in delayed CADR.

Based on our interest on Immunology and ACD and the previous experience of our group on studying the *in vitro* stimulating effect of contact sensitizers on antigen presenting cells, we evaluated the effect of systemic drugs on dendritic-

like cells *in vitro*, which may similarly contribute to enhance their presentation to the immune system.

Having a very solid area of Dermatopathology in our Department and having to deal frequently with severe patients with CADR in our ward, we characterized histopathology of some severe reactions in order to better understand pathophysiologic mechanisms underlying the skin aggression and, eventually, establish diagnostic or prognostic markers.

Evaluation of susceptibility factors might also represent a step further in defining patients at risk and, therefore, preventing the more severe and life-threatening CADR, often induced by non-essential drugs, as allopurinol. For this we had the help of the Histocompatibility centre, with which we had previously collaborated on the immunological and genetic study of many patients.

Therefore, with the available resources we combined the knowledge of ACD and patch testing, Immunology, particularly of dendritic cells (DC), Dermatopathology and HLA genotyping with the daily clinical experience with patients suffering CADR. We investigated some aspects of delayed CADR, in order to contribute to a better knowledge of their pathophysiology and, therefore, have a better capacity to solve the challenging cases frequently observed at the Department of Dermatology.

B.
IMMUNE MEDIATED CUTANEOUS ADVERSE DRUG REACTIONS: THE STATE OF THE ART

B. IMMUNE MEDIATED CUTANEOUS ADVERSE DRUG REACTIONS: THE STATE OF THE ART [1]

B.1. Definition and main pathophysiological mechanisms

According to the definition of the World Health Organization (WHO), an adverse drug reaction (ADR) is "a response to a medicine which is noxious and unintended and which occurs in doses usually used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function" (Edwards and Aronson 2000). The majority of ADR are expected, dose dependent and represent an exaggerated pharmacological effect of the drug. About a fifth of the ADR are unexpected, idiosyncratic and dose independent. They are considered type B or "bizarre", opposing the previous type A or "augmented". Other types of ADR may be defined as type C or chronic and cumulative, type D or delayed, including carcinogenesis and teratogenesis, type E or event on drug withdrawal, and type F or drug failure (Edwards and Aronson 2000),(Friedmann 2003).

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[1] This chapter represents a revised and significantly extended version of a book chapter we wrote for the 7th and 8th Edition of Dermatotoxicology, upon invitation by Professor Derk Bruynzeel:

1. Mechanisms in cutaneous drug hypersensitivity reactions (Chapter 9). M Gonçalo, D Bruynzeel *in* Dermatotoxicology. Wilhelm K-P, Zhai H, Maibach HI (Eds). 8th Ed. CRC Press. Boca Raton, 2012: 78-92. *ISBN 978184184556; eBook ISBN:9781841848570*
2. Mechanisms in cutaneous drug hypersensitivity reactions (Chapter 27). M Gonçalo, D Bruynzeel *in* Marzulli and Maibach's Dermatotoxicology. Zhai H, Wilhelm KP, Maibach HI (Eds) 7th Ed. CRC Press. Boca Raton, 2008, 259-268

According to the general ADR classification, most cutaneous adverse drug reactions (CADR) can also be predictable or expected (type A), or belong to types C to F. Unpredictable, idiosyncratic CADR (type B), although less frequent, are those that raise more questions concerning their clinical recognition, aetiological diagnosis and appropriate therapy.

Most CADR of the B type, or drug eruptions, represent an acquired and specific hypersensitivity reaction to the drug. All the four classical mechanisms of immune hypersensitivity, defined by Gell and Coombs, can be involved in the immune response to drugs.

In type I, or immediate hypersensitivity, the drug is recognized by an immunoglobulin (Ig) E and induces degranulation of IgE laden mast cells or basophils, causing urticaria, angioedema or anaphylaxis. In type II hypersensitivity reactions, complement-fixing antibodies (IgG or, rarely, M) recognize the drug coupled to a cell surface and cause antibody-dependent cellular cytotoxicity (ADCC), as in drug-induced haemolytic anaemia or thrombocytopenia. In type III group of hypersensitivity reactions, IgG or IgM antibodies recognize a soluble antigen, form immune complexes that activate complement (C3a/C5a) and induce aggression of small vessel walls, causing leukocytoclastic vasculitis (Friedmann 2003),(Pichler 2007).

Delayed type IV hypersensitivity that involves specific effector T cells has been documented in several delayed patterns of CADR. T cells recognize the drug or a metabolite coupled to small peptides, nevertheless, different subsets of drug specific T cells and soluble effectors participate in the inflammatory response and, consequently, cause different patterns of delayed drug eruptions. The most frequent ones are the maculopapular exanthema, drug reaction with eosinophilia and systemic symptoms (DRESS) or drug induced hypersensitivity syndrome (DIHS), acute generalized exanthematous pustulosis (AGEP), Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) and fixed drug eruption (Lerch and Pichler 2004),(Pichler et al. 2011),(Pirmohamed et al. 2011).

Apart from the four classical types of hypersensitivity defined by Gell and Coombs, the drug can modify the immune response promoting autoimmunity or induce the production of pathogenic antibodies directed against skin structures, as in vancomycin-induced linear IgA dermatitis (Coelho et al. 2006), in D-penicillamine-induced pemphigus (Allanore and Roujeau 2007) or in terbinafine and esomeprazol-induced subacute lupus erythematosus (Farhi et al. 2006),(Dam and Bygum 2008).

For a better clinical and pathophysiological understanding, the main immune mediated drug eruptions are divided into immediate and nonimmediate or delayed CADR. Although the time frame of these two types of drug reactions is not universally accepted (Romano et al. 2012), this division has, nevertheless, very important implications for the decision on the proper diagnosis and therapy, identification of the culprit drug and decision on the most adequate diagnostic tests to confirm its aetiology.

B.2. Immediate cutaneous adverse drug reactions

Immediate adverse skin reactions can be mild and localized, as in urticaria, but they can generalize and progress rapidly to facial, laryngeal or upper airway angioedema with respiratory distress (Quincke angioedema) or to life-threatening anaphylaxis. Reactions develop in minutes or within the first hour after drug exposure, according to ENDA (European network on drug allergy) (Romano et al. 2005),(Torres et al. 2007),(Hjortlund et al. 2013), or up to 6 hours of drug intake (Pichler 2007). They occur mostly on intravenous or oral exposure although other routes, including skin or mucosal exposure can also induce the reaction (Krautheim et al. 2004).

In immediate CADR the antigen is usually coupled with aminoacids, forming a haptenized protein, which is recognized by the antigen-binding fragment (Fab) of the IgE. This immunoglobulin is bound to the surface of tissue mast cells and circulating basophils through the type 1 high affinity receptor for IgE, FcεRI. When the antigen (drug + protein) bridges two or more IgEs on the cell surface, an intracellular cascade of events is activated in mast cells and basophils. Intracellular granules migrate to the cell periphery, fuse their membranes with the cell membrane and preformed inflammatory mediators contained in the granules, like histamine, tryptase, heparin, cytokines and chemokines, are released to the extracellular space. Moreover, secondary vasoactive mediators are produced after activation of membrane phospholipids, namely prostaglandins, leukotrienes and platelet activation factor (PAF). Together, these mediators induce vasodilatation, increase vascular permeability and cause pruritus, as in urticaria and angioedema (Fig. B.1).

In more generalized and severe forms, mediators liberated also from circulating basophils induce associated systemic symptoms of anaphylaxis, like bronchospasm, hypotension and bradycardia (Blanca et al. 2009).

A non-specific mast cell/basophil degranulation can occur in pseudo-allergic reactions that are sometimes difficult to distinguish from allergic, IgE-mediated reactions.

Drugs inducing IgE dependent reactions are mainly the penicillins, cephalosporins, fluorquinolones, radiologic contrast media, neuromuscular blockers and some NSAIDs, although the latter often induce non-specific, non-IgE dependent mast cell degranulation.

In the immediate reaction a massive mast cell and basophil degranulation may be life threatening, therefore it needs prompt intervention with i.v. corticosteroids and, eventually, adrenaline. Serum levels of tryptase may confirm mast cell and basophil granule release in the acute stage (Lee et al. 2013b), but it is usually necessary to act before any laboratory result is available.

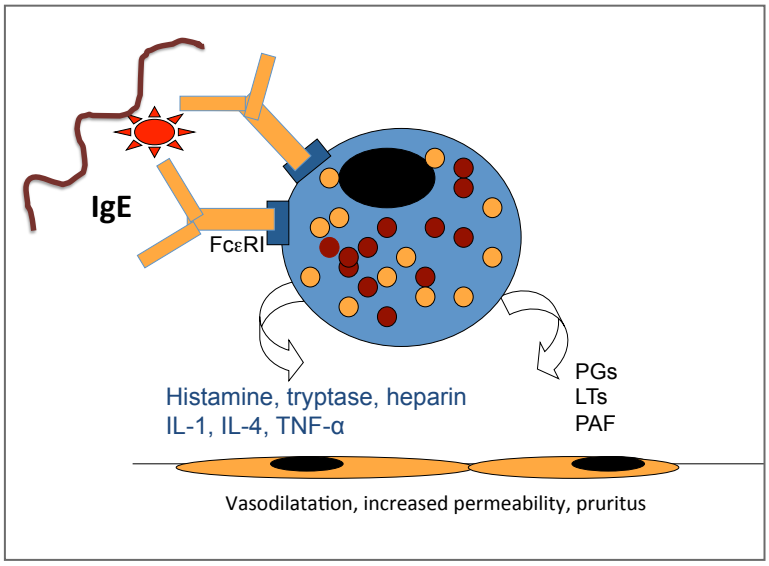


Figure B.1 – Mechanism of immediate drug reactions. The drug combined with a peptide is recognized by the Fab of two IgEs fixed on mast cells and causes mast cell degranulation with liberation of pre-formed mediators (histamine, tryptase, heparin and several cytokines and chemokines). Secondary mediators are formed upon activation of membrane phospholipids (prostaglandins, leukotrienes and platelet activating factor-PAF). Together, they induce vasodilation, increase vascular permeability and cause pruritus, as observed in urticaria.

Clinical patterns of immediate CADR are usually easy to distinguish from delayed reactions, but an exact distinction based only on the time frame of 1 hour after drug administration established by the ENDA group (Romano et al. 2005),(Torres et al. 2007) is not the definitive answer, especially if the clinical presentation is not taken into account (Hjortlund et al. 2013). In immediate CADR, apart from the timing, the confirmation of diagnosis of the culprit drug relies both in *in vitro* and *in vivo* diagnostic tests.

I*n vitro* tests detect drug specific IgE by immunoassays, namely radioallergosorbent test (RAST), enzyme linked immunosorbent assay (ELISA) and CAP-immunoassay/capture fluorescence enzyme immunoassay (CAP-FEIA), or evaluate drug specific basophil activation. Basophil tests measure either the increase in the surface expression of CD63 or CD203c, evaluated by flow cytometer, or the intensity of mediator release, induced upon *in vitro*

exposure to the culprit drug (Kvedariene et al. 2006),(Blanca et al. 2009),(Ebo et al. 2011).

In vivo tests include skin testing (prick or intracutaneous tests with immediate readings) and, eventually, drug rechallenge under medical supervision (Demoly 2005).

B.3. Delayed cutaneous adverse drug reactions: main clinical patterns

Nonimmediate or delayed drug eruptions begin usually within a few days or weeks after drug intake or, exceptionally, within a few hours in previously sensitized patients. These delayed CADR represent a drug-specific T cell reaction and manifest under a wide spectrum of clinical patterns, usually a generalized symmetrical maculopapular or more urticarial exanthema, with or without target-like lesions, bullae or pustules. The combination of these different elementary lesions, the histopathology, the association with systemic symptoms and their evolution, allow the definition of the more common phenotypic patterns of nonimmediate drug eruptions. Some are more generalized like the maculopapular exanthema, DRESS/DIHS, AGEP and SJS/TEN, whereas others are usually more localized, like the fixed drug eruption (Roujeau 2005), (Pirmohamed et al. 2011).

B.3.1. Maculopapular exanthema

Maculopapular exanthema, the most frequent pattern of CADR, appears as a generalized symmetric eruption of isolated and confluent erythematous macules and/or papules, often starting in the trunk and spreading to the extremities (Fig. B.2). Mucosae are not affected and systemic symptoms are

mild (malaise and low-grade fever). The reaction develops within 7-14 days after drug intake or within 1 or 2 days in previously sensitized patients. Maculopapular exanthema progresses for a few days, even after drug suspension, and fades progressively within 10-15 days, often with desquamation.

Maculopapular exanthema is mainly induced by antibiotics (aminopenicillins, cephalosporins and sulfonamides), allopurinol and anticonvulsants (Roujeau 2005),(Yawalkar 2005),(Pirmohamed et al. 2011). It may be difficult to distinguish a drug-induced maculopapular exanthema from a viral exanthema or other causes of exanthema, both clinically and on histopathology (Naim et al. 2011),(Bellini et al. 2013),(Seitz et al. 2013).

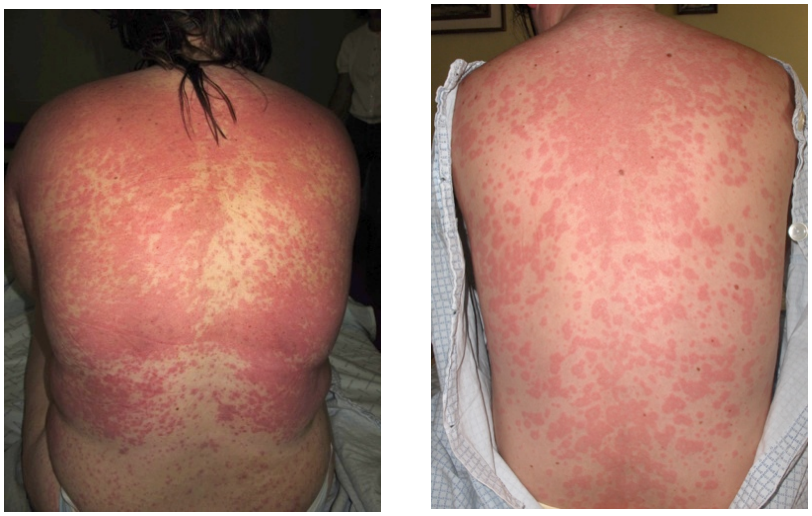


Figure B.2 – Maculopapular exanthema from cotrimoxazole (left) and from amoxicillin (right).

Histology of early macular or papular lesions of maculopapular exanthema shows interface dermatitis with vacuolar degeneration of basal keratinocytes, mild lymphocyte exocytosis and spongiosis in the lower epidermal layers, and scattered apoptotic keratinocytes. Lymphocytes are observed mainly at the dermal-epidermal junction and papillary dermis, often around dilated blood

vessels. Eosinophils, and occasional neutrophils, occur in the dermis or inside dermal vessels (Pichler et al. 2002),(Naim et al. 2011),(Seitz et al. 2013).

Skin infiltrating lymphocytes are skin homing, highly activated T cells, expressing high quantities of adhesion molecules and chemokine receptors. Most are $CD4^+$ and $CD8^+$ T cells, with $CD8^+$ T cells found mainly in the epidermis. $CD8^+$ T cells are also the main infiltrating cell in maculopapular exanthema HIV infected patients (Pichler et al. 2002),(Pirmohamed et al. 2011),(Brönnimann and Yawalkar 2005),(Yawalkar 2005), explaining the occurrence of CADR in Acquired Immunodeficiency Syndrome (AIDS), even when $CD4^+$ count is very low.

Both $CD4^+$ and $CD8^+$ T cells, but particularly $CD8^+$ T cells, NK and NKT cells, express high levels of the cytotoxic mediators perforin, granzyme B and granulysin (Schlapbach et al. 2011b).

Keratinocytes show signs of stimulation, particularly at the basal layer. Apart from the regular expression of HLA class I molecules, they also express HLA class II and adhesion molecules. Langerhans cells, other dendritic cells and $CD68^+$ macrophages are also increased in the dermis and express high levels of HLA-II (Pichler et al. 2002), suggesting their possible commitment to antigen presentation.

B.3.2. Drug reaction with eosinophilia and systemic symptoms (DRESS)/Drug-induced hypersensitivity syndrome (DIHS)

DRESS/DIHS is a severe life-threatening CADR, described in the early 30's in relation with phenytoin, and recognized initially under the designation of anticonvulsant hypersensitivity syndrome (Husain et al. 2013). In the 90's Bocquet et al. proposed the term DRESS, for "drug rash with eosinophilia and systemic symptoms", and defined the first criteria for this entity. Later, as cutaneous manifestations could be absent, the meaning of the letter "R" in the

acronym was replaced by reaction. Diagnostic criteria were recently and independently revised by a Japanese consensus group, which mainly uses the designation DIHS (Shiohara et al. 2007), and the European multicentre group studying severe CADR, the RegiSCAR (Kardaun et al. 2007),(Cacoub et al. 2011).

The clinical presentation of DRESS is heterogeneous, usually combining cutaneous and systemic involvement. It begins with fever (>38-39ºC) and malaise, followed by a severe maculopapular exanthema or a generalized exfoliative dermatitis (Husain et al. 2013),(Ogawa et al. 2013) (Fig. B.3), often mistaken for an infectious disease at its onset (Lee et al. 2012a). Severe facial edema with vesicles or pustules is frequently associated with the exanthema. Atypical target lesions, purpura, vesicles, pustules or bullae can also occur (Cacoub et al. 2011),(Walsh et al. 2013),(Husain et al. 2013). The skin reaction in DRESS may be clinically similar to a maculopapular exanthema, and only the association of systemic symptoms and their severity may allow the diagnosis of DRESS.

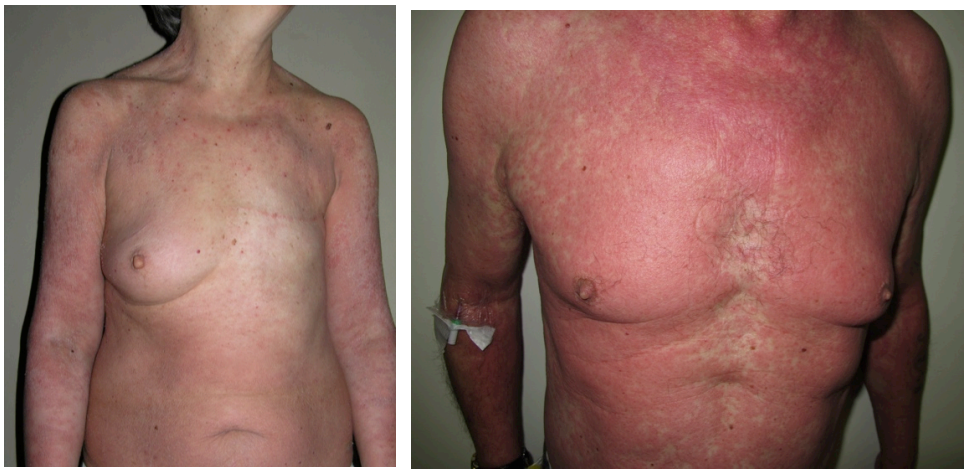


Figure B.3 – Skin manifestations in DRESS. Exfoliative erythroderma from carbamazepine (left). Maculopapular exanthema with confluent lesions from allopurinol (right).

Leucocytosis with circulating atypical (activated) lymphocytes and eosinophilia (>700 eosinophils/µl or >10% of circulating leucocytes) are frequently observed, with eosinophil counts often increasing only late in the course of DRESS (Shiohara et al. 2007),(Santiago et al. 2010),(Descamps and Ranger-Rogez 2014).

Most affected internal organs are the liver (severe hepatitis and/or cholestasis), lymph nodes (tender lymphadenopathy), kidney (renal failure), heart (myocarditis), blood (hemophagocytic syndrome), digestive tract and/or lung (Descamps et al. 2010),(Husain et al. 2013),(Descamps and Ranger-Rogez 2014).

DRESS has a delayed onset, usually more than 2 weeks and up to 6-8 weeks after the initiation of the culprit drug. It is induced mainly by an anticonvulsant, allopurinol, sulfasalazine, abacavir, nevirapine, dapsone or minocycline.

DRESS also regresses slowly (usually >3 weeks) and may have a prolonged course (>2 months), often with exacerbations, either related with steroid withdrawal, viral reactivation or administration of a related or non-related drug (Kano et al. 2004),(Hirahara et al. 2010),(Descamps and Ranger-Rogez 2014).

DRESS patients can suffer late complications, namely thyroiditis, adrenal insufficiency, diabetes insipidus and other autoimmune manifestations (Cookson et al. 2013),(Descamps 2013), end-organ failure, as chronic renal failure following allopurinol-induced DRESS (Chen et al. 2013), or reactivation of varicella-zoster virus with severe herpes zoster (Kano et al. 2012).

This particular type of drug-induced reaction seems to be highly dependent on herpes virus infection or reactivation, particularly HHV-6, EBV, CMV or HHV-7. Actually, the Japanese team added viral reactivation to the DRESS criteria (Shiohara et al. 2007). Nevertheless, reinforcing the participation of the drug, positive patch tests with a very low concentration can be observed later,

particularly when carbamazepine or abacavir are the culprit drug (Santiago et al. 2010),(Phillips and Mallal 2009a).

In DRESS, $CD4^+$ and $CD8^+$ T cells infiltrate the dermis and epidermis with a lichenoid pattern, a more spongiotic pattern, an erythema multiforme-like pattern (Naisbitt 2004),(Hashizume and Takigawa 2005),(Walsh and Creamer 2011) or, often, with a dense dermal-epidermal infiltrate mimicking mycosis fungoides. This lymphomatous infiltrate, in the skin and in lymph nodes, has suggested a previous designation of drug-induced pseudolymphoma (Husain et al. 2013). Eosinophils can infiltrate the skin, particularly in the perivascular dermis, and epidermal changes like basal cell vacuolization, scattered apoptotic keratinocytes and lymphocyte exocytosis are often observed (Chen et al. 2010),(Walsh et al. 2013), as in maculopapular exanthema (Naim et al. 2011).

B.3.3. Acute generalized exanthematous pustulosis

AGEP is a very peculiar reaction pattern induced by drugs in more than 90% of cases, mainly aminopenicillins and other antibiotics (pristinamycin). It is also frequently associated with diltiazem and terbinafine (Pirmohamed et al. 2011), but seldom related with allopurinol and anticonvulsants (Sidoroff et al. 2007).

AGEP is characterized by the acute onset of a symmetrical widespread edematous erythema covered by small nonfollicular sterile pustules, predominating in the large body folds, high fever (>38ºC), leucocytosis, neutrophilia and, occasionally, eosinophilia (Fig. B.4).

The reaction develops within 1 week of drug intake and usually regresses in 5-10 days after drug withdrawal (Sidoroff 2012), but prolonged and more severe courses can be observed, often with overlapping features with TEN (Peermohamed and Haber 2011).

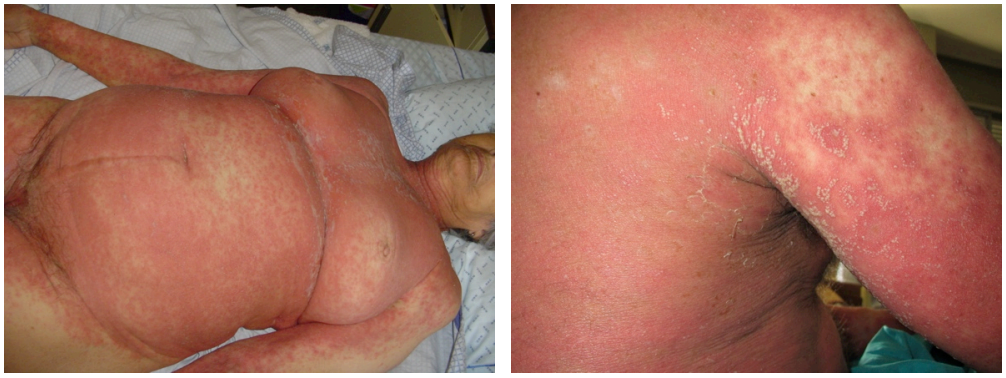


Figure B.4 – Two cases of **acute exanthematous generalized pustulosis** induced by ciprofloxacin (left) and acyclovir (right), with pustules predominating in large body folds.

Early biopsies from AGEP show a dermal-epidermal infiltration of T cells, mainly $CD4^{+}HLA\text{-}DR^{+}CD25^{+}$ T cells, with discrete vacuolar keratinocyte degeneration and a perivascular infiltrate of lymphocytes and eosinophils, sometimes with vasculitis (Sidoroff et al. 2001),(Britschgi and Pichler 2002). Lesions progress to spongiotic vesicles that, due to neutrophil accumulation, soon transform into epidermal and subcorneal spongiform pustules, the histologic hallmark of AGEP (Britschgi and Pichler 2002),(Schaerli et al. 2004),(Halevy et al. 2010).

B.3.4. Stevens-Johnson syndrome/Toxic epidermal necrolysis

CADR with epidermal necrolysis represent a spectrum of life-threatening diseases, characterized by full thickness epidermal and mucosal necrosis that progresses to epidermal sloughing, with a increasing extension from SJS to TEN.

TEN, also known as Lyell's Syndrome, was probably first described by Debre *et al.,* in 1939 (Schwartz et al. 2013a). In 1956, Alan Lyell reported 4 cases with

acute necrosis and detachment of the skin and mucous membranes under the designation of toxic epidermal necrolysis (Roujeau et al. 2011). The first cases of SJS were reported in children by two American physicians, Albert Stevens and Frank Johnson, in 1922, as a "new eruptive fever associated with stomatitis and ophtalmia" (Schwartz et al. 2013a).

TEN is still occasionally treated as a separate entity from SJS (Schwartz et al. 2013a), but since 1993 it is more consensual to consider both entities as two extremes of an identical process that differ in the body surface area (BSA) of necrolysis: more than 30% in TEN, 10-30% in the SJS/TEN overlap syndrome and 1%-10% in SJS. A form of TEN without spots, with more than 10% skin detachment was also individualized (Bastuji-Garin et al. 1993). To include this whole overlapping spectrum that frequently progresses from SJS to SJS/TEN overlap and to TEN, Roujeau and co-workers recently suggested the designation of exanthematic necrolysis (Roujeau et al. 2011).

These entities should be distinguished from erythema multiforme, particularly from its major variant, characterized by mucosal erosions associated with typical or atypical non-confluent and raised target-like lesions, with <1% skin detachment. Erythema multiforme major is more often associated with infections, namely by Herpes simplex virus or *Mycoplasma pneumonia*, than it is induced by drugs (Mockenhaupt 2011).

SJS/TEN are rare forms of CADR, with one case of TEN occurring in average per year and per one million inhabitants. The number may rise up to 2-7 cases if the whole spectrum of SJS/TEN is considered. A culprit drug is identified in more than 90% of cases, usually an antibiotic (sulfonamide), allopurinol, an anticonvulsant (lamotrigine, carbamazepine), nevirapine or a NSAIDs (oxicam) (Allanore and Roujeau 2007).

Fever, malaise and painful mucosal erosions often occur before the onset of the skin eruption, which is then associated with other systemic symptoms. Widespread symmetrically distributed and confluent macular lesions with vesicles or bullae, and isolated atypical flat targets at the periphery, constitute the initial skin presentation of SJS/TEN. Exanthema progresses from the face and upper trunk to the extremities and often affects the palms and soles. In TEN, flaccid bullae develop and coalesce to form large blisters (Fig. B.5-7).

Necrotic epidermis is easily detached from the dermis by lateral pressure (Nikolsky's sign), showing a denuded and painful dermis. Epidermal sloughing may progress quickly (<24h) or more slowly, in this case reaching the final area of skin detachment within a few days to a week.

Conjunctivae, oral and genital epithelial shedding is usually intense and painful and tends to form pseudomembranes and mucosal adhesions. Necrosis of the epithelium of the oropharynx, gastrointestinal tract, trachea and bronchia can be associated.

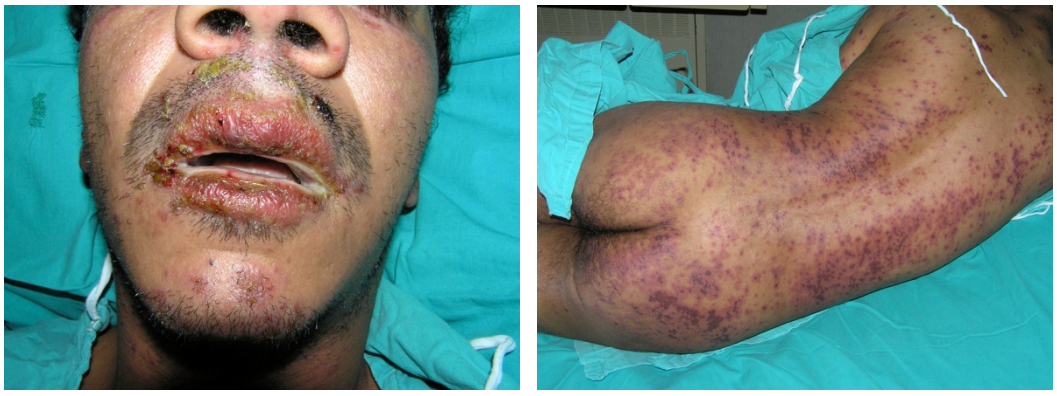


Figure B.5 - Stevens-Johnson's syndrome (SJS) induced by nevirapine. Severe oral erosions and an exanthema with atypical flat targets and 2% of BSA of epidermal necrolysis.

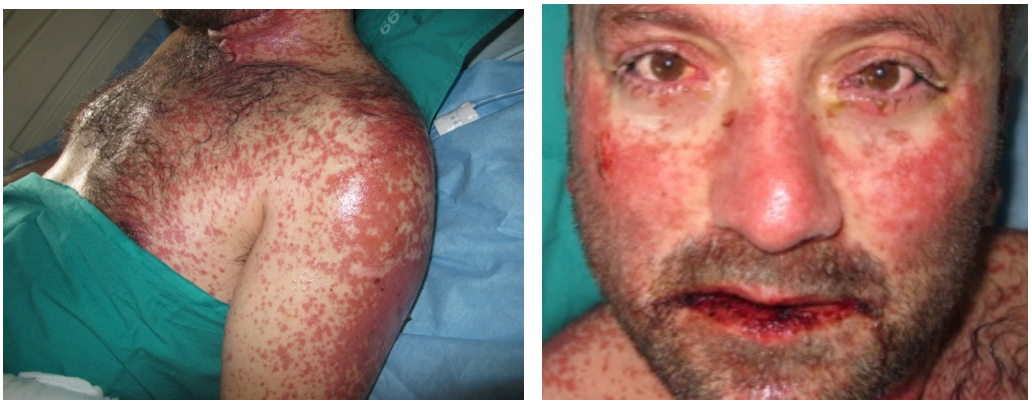


Figure B.6 – Overlap Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) induced by allopurinol. Severe ocular and oral erosions and skin necrolysis affecting more than 10% of BSA.

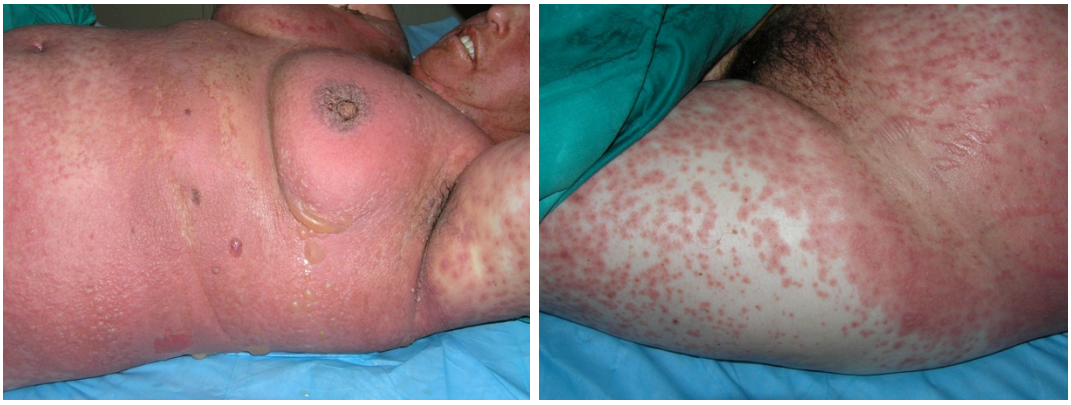


Figure B.7 - Toxic epidermal necrolysis (TEN) in a patient treated with carbamazepine after surgery for meningioma. Extensive areas of flaccid bullae and atypical targets at the periphery of the areas with confluent epidermal necrolysis.

Mortality can be high in SJS/TEN, either due to acute skin failure, systemic manifestations (lung, kidney) and/or secondary complications (Pinheiro et al. 2013). The SCORTEN (severity-of-illness score for TEN), performed at the date of admission and within 2 days, is a scoring system that has been validated as a good prognostic index. It scores one point for each of the following items: patient's age above 40 years, co-existence of malignancy, skin sloughing above 10 % of the total body surface area, heart rate > 120/min, blood sugar > 250

mg/dL, serum bicarbonate > 20 mEq/L and serum urea nitrogen > 27 mg/dL. The higher the number of parameters present (from 0 to 7) the worse the prognosis, with an expected mortality below 15% for a score 2 and over 90% when 5 or more criteria are present (Faye et al. 2005),(Valeyrie-Allanore et al. 2013).

After discharge and apparent resolution of SJS/TEN, mortality rate is still increased within the next 3 months to one year. This occurs even in the less severe forms of SJS and most particularly in older patients (>54) with additional comorbidities or recent malignancy (Sekula et al. 2013).

Those who survive SJS/TEN often have muco-cutaneous sequelae, mostly ocular complications like dry eye syndrome, corneal opacity, trichiasis, symblepharon or even blindness. Oral and genitourinary complications can also occur, namely xerostomia, dental and periodontal disease, oral synechiae, ageusia, vulvovaginal stenosis and/or synechia and phimosis, as well as cutaneous scaring and dyspigmentation (Schwartz et al. 2013b).

The most striking histologic marker of SJS/TEN is the confluent keratinocyte cell death (apoptosis) that may extend to all epidermal layers. The intensity of the inflammatory infiltrate ranges from almost absent to a dense dermal T infiltrate. According to some studies, the intensity of the infiltrate seems to correlate positively with the BSA of skin detachment and, very probably, with the mortality rate, but results are not fully concordant (Quinn et al. 2005),(Faye et al. 2005).

$CD4^+$ and $CD8^+$ T cells are scattered in the dermis and in the epidermis, but most lymphocytes that infiltrate the lesions are usually found in the blister fluid. They are mostly activated cytotoxic $CD8^+CD56^+$ T cells, NK and NKT cells rich in granulysin (Correia et al. 1993),(Nassif et al. 2004a),(Roujeau et al. 2011),(Teraki et al. 2013).

Contrasting with a reduction of $CD1a^+$ Langerhans cells, Factor $XIIIa^+$ dermal dendritic cells are increased, as well as activated monocytes that express high

levels of CD80/CD86 and CD137L, molecules that are highly involved in T cell activation (Tohyama and Hashimoto 2012).

B.3.5. Fixed drug eruption

Fixed drug eruption is a particular drug hypersensitivity reaction involving the skin and mucosa. It occurs as round erythematous or more violaceous macules or plaques, some with central bulla and epidermal detachment. Lesions regress spontaneously within 10-15 days with a grey-brown hyperpigmentation, and recur on the same place within hours after drug reintroduction, often with new lesions (Fig. B. 9).

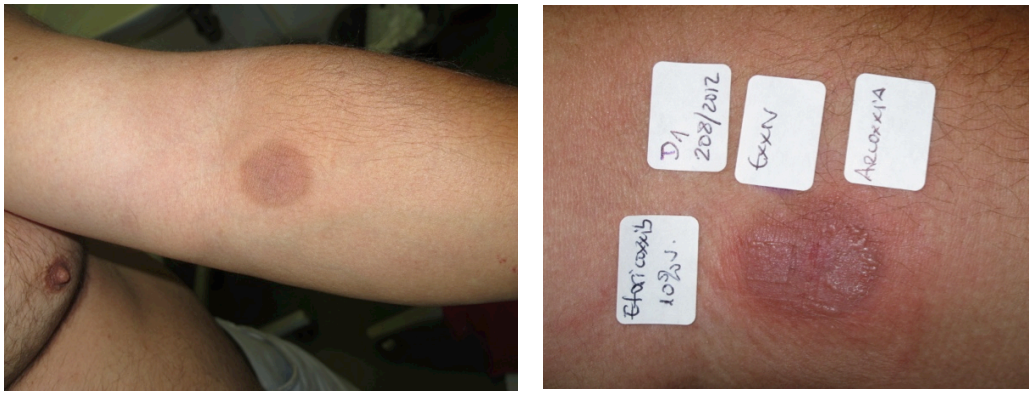


Figure B.9 - Single residual round, pigmented macule from an etoricoxib-induced **fixed drug eruption** (left). Reactivation of the lesion during a lesional patch test using 2 preparations containing etoricoxib diluted at 10% in petrolatum, using the powder from the pills of two commercialized medicaments containing this NSAID (Arcoxia®, Exxiv®) (right).

Lesion number and size may vary from a few small to many large and coalescent lesions with a more widespread involvement, difficult to distinguish from TEN without spots (Shiohara and Mizukawa 2012). Nevertheless, in fixed

drug eruption distribution of lesions is more asymmetric, there are no systemic symptoms and the eruption resolves rapidly (Allanore and Roujeau 2007) (Fig. B. 10).

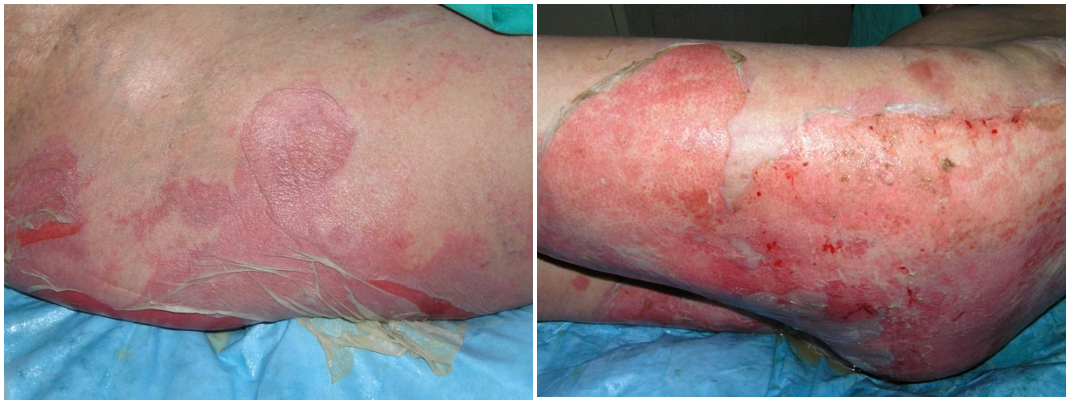


Figure B.10 – Generalized fixed drug eruption induced by a NSAID, simulating TEN, but with very well limited and large round areas with epidermal sloughing with normal skin between the bullous lesions (left). Almost complete healing within a few days (right) and absence of atypical targets in normal skin, are arguments that favour the diagnosis of fixed drug eruption.

At the acute phase, skin histology shows a lichenoid reaction with mononuclear inflammatory infiltrate, mainly at the dermal epidermal junction, vacuolization of basal keratinocytes and scattered or more extensive keratinocyte apoptosis, eventually involving the whole epidermal thickness, as in TEN (Shiohara and Mizukawa 2012).

Upon regression, melanophages accumulate in the dermis and $CD8^+$ T cells persist in the epidermis, in abnormal numbers and for many years after clinical resolution of the eruption (Shiohara et al. 2002). These are resting drug specific $CD8^+$ T cells that are easily activated when the culprit drug reaches the epidermis again, either by systemic or epicutaneous exposure. This explains frequent positive epicutaneous tests, only on residual lesions, within a few hours of contact with the relevant drug. Moreover, these positive lesional patch tests show histopathological features that are typical of the acute fixed drug

eruption, namely an inflammatory infiltrate with keratinocyte apoptosis and vacuolization of basal cell keratinocytes (Gonçalo et al. 2002),(Andrade et al. 2011).

B.4. Pathophysiologic mechanisms in delayed cutaneous adverse drug reactions and allergic contact dermatitis

Pathophysiologic mechanisms involved in delayed immune mediated CADR are very complex. Many recent studies have addressed these questions, but there is still much to be investigated and it is not easy to conduct such research. The CADR is often the result of many concurrent factors apart from the drug, namely other concomitant drugs, viral infections or chronic immuno-inflammatory disease. There is no reliable animal model to study all the reactions (Rozières et al. 2010),(Saito et al. 2013) and investigation has to rely mainly on human data, mostly during the acute phase of the reaction, and on ex-vivo studies with cells isolated from patients. Moreover, there are many clinical patterns and many drugs involved, each one with its specific features, which makes this subject even more complicated but, on the other hand, more fascinating.

As previously referred, there are many similarities, both at the clinical and pathophysiological level, between CADR and ACD. The latter represents the classical pattern of a delayed type hypersensitivity reaction in the skin, in this case to the epicutaneous application of small chemicals, e.g. topical drugs. Much has been learned recently on the pathophysiologic mechanisms involved in ACD. Both human studies and, particularly, animal research has given new insights into the mechanisms of antigen presentation, T cell sensitization and effector mechanisms (Martin et al. 2010),(Peiser et al. 2012),(Honda et al. 2013). A significant amount of knowledge concerning the pathophysiology of CADR has been inferred by analogy with ACD, and skin reactivity to drugs in

patch tests has also been used as an important tool to characterizing cells involved in CADR.

The contribution to the understanding and study of these two delayed cutaneous reactions is reciprocal. For instance, diagnostic tests developed for the diagnosis of delayed CADR, like lymphocyte activation tests and the ELISpot (enzyme-linked immunospot), are increasingly being studied in ACD (Spiewak et al. 2007),(Bordignon et al. 2008),(Lindemann et al. 2008),(Nosbaum et al. 2009), although they are still very far from standardization and clinical applicability.

B.4.1. Pathophysiologic mechanisms in allergic contact dermatitis

ACD presents usually as pruritic eczema beginning at the area of contact with the offending chemical. In the acute reaction, erythema, papules and vesicles, sometimes with exudation, appear within 12-72 hours after contact and may progress to crusting, scaling and, in chronic phases, to lichenification.

On histology there is vasodilation of papillary capillaries with dermal edema and a perivascular lymphomononuclear infiltrate, but main changes occur mostly at the epidermis: lymphocyte exocytosis, occasional keratinocyte apoptosis, exoserosis and spongiosis with liquid accumulation between the keratinocytes that leads to rupture of desmosomes and vesicle formation.

Infiltrating cells are mostly T cells, but monocytes, DCs and, transiently, neutrophils are also observed (Sebastiani et al. 2002). Lymphocytes are mainly $CD8^+$ and $CD4^+$ T cells, T helper (Th) 1 or T cytotoxic (Tc) 1 cells, but Th2/Tc2 and, particularly Th17, are increasingly being described, as well as regulatory T cells (Treg) (Martin et al. 2010),(Pennino et al. 2010),(Honda et al. 2013).

Keratinocytes show signs of activation, expressing high levels of HLA-class II and adhesion molecules, namely intercellular adhesion molecule (ICAM-1/CD54). Vascular endothelial cells also express high levels of adhesion

molecules to guide inflammatory cells to transmigrate to the skin. Some subsets of DC may be reduced whereas other are highly activated and interact locally with T cells. Other effector cells, including NK and NKT cells are also observed, as well as monocytes, fibroblasts, neutrophils and mast cells (Honda et al. 2013),(Chong et al. 2014).

Apart from the eczematous reaction, different clinical and histopathology patterns have been recognized in ACD, namely pustular, lichenoid, erythema-multiforme-like, granulomatous, purpuric, pigmented, and lymphomatoid ACD (Goon and Goh 2011). They are very probably dependent on the predominance of a particular subtype of effector antigen specific T cells infiltrating the skin: $CD4^+$ or $CD8^+$ T cells, Th1, Th2, Th17 or Tc1 and Tregs.

Chemicals that cause ACD, like nickel, para-phenylenediamine, fragrance chemicals, preservatives or drugs, are small reactive chemicals with less than 500 Da, therefore they are not complete antigens and are usually referred as haptens. They easily penetrate the horny layer and combine with aminoacids from skin proteins (lysine, histidine, cysteine) to become a complete antigen. Some are active on their own, the haptens, but others behave as prohaptens or prehaptens, as they need previous modification into the reactive chemical, respectively by enzymatic metabolic processes (cytochrome P450 enzymes) or by nonenzymatic reactions (auto-oxidation). The combination of the reactive chemical and the peptide will form the antigen to be recognized by the T cell (Lepoittevin 2011).

In the immune process in ACD, two phases are usually considered. A clinically silent induction or sensitization phase, that in man lasts 10-14 days to many years. It represents the attempts of the new chemical to be presented and recognized by the immune system eventually reaching the situation where the immune system no longer “tolerates” the presence of this chemical, recognizes

it as an “harmful” non-self chemical and creates a specific immune response to eliminate it from the body.

This process can be very quick and almost universal for strong sensitizers, like DNCB (dinitrochlorobenzene), that induces a specific immune response in almost every individual, as long as sufficient exposure occurs (Friedmann 2007). For other allergens, the sensitization process can oblige to many and prolonged exposures, it may need additional stimulus to develop and occurs only in a limited number of individuals.

Once an individual becomes sensitized, meaning he has a significant number of antigen specific effector and memory T cells that can reach the skin, a further contact with the antigen, or a chemically related substance, may induce a clinically visible reaction, an ACD. This is the elicitation or effector phase. It occurs usually within 72 hours after exposure to the culprit chemical, but its time-course depends on the exposure dose/skin area and the intensity of the previous sensitization process (Friedmann 2007). Skin cells respond to the presence of the chemical, recruit and activate specific T cells and other effector cells, which, together, induce an inflammatory reaction to eliminate the allergen.

B.4.1.1. The sensitization phase

During the sensitization phase in ACD, the antigen is processed by skin dendritic cells and then presented to naïve T cells in the regional lymph node.

Skin resident Langerhans cells that form a continuous network within the epidermis, or other immature skin DC, particularly Langerin^{+} dermal DC, are usually very efficient in the capture and processing of antigens. They capture antigens, mostly by endocytosis or related processes, and, after a complex intracellular processing, combine the active epitopes with HLA molecules that will be transferred to the surface of the DC.

These skin DC are usually in an immature state concerning their capacity to present antigens to T cells. Therefore, for an effective presentation, they have to maturate, that is, they have to upregulate the expression of HLA molecules and of adhesion and costimulatory molecules and have to produce cytokines that are necessary for communicating with T cells and, eventually, activating them. Simultaneously, skin DC have to change the repertoire of chemokine receptors in order to migrate out of the skin, towards the draining lymph nodes, in search for a naïve T cell with a TCR that might recognize the antigen they carry.

Contact sensitizers, as reactive chemicals, induce several non-specific inflammatory responses in epidermal and, eventually, also in dermal cells, a xenoinflammation that is crucial for the maturation and migration of skin DC, and, consequently, to the sensitization process. Actually, contact haptens trigger responses very similar to microbial products and induce an innate immune response that is important to call upon the elements of the adaptive immunity. Some haptens mimic PAMP (pathogen associated molecular patterns) and use PRR (pattern recognition receptor) common to microbes. For instance, nickel, palladium and cobalt dimerize and activate the Toll-like receptor (TLR) 4, the PRR receptor for the lipopolysaccharide of $Gram^{-}$ bacteria (LPS) (Rachmawati et al. 2013).

Other haptens induce the production of endogenous danger signals (DAMP-damage-associated molecular patterns), namely reactive oxygen species (ROS), extracellular adenosine triphosphate (ATP) or hyaluronic acid fragments (HAF), which are also recognized by receptors innate immunity.

ROS, HAF and ATP activate either the membrane associated TLR4/TLR2 or the P2X7 purinergic receptor, the cytoplasmic nucleotide-binding domain (NOD)-like receptor or the Keap1-Nrf2-ARE pathway (Kelch-like ECH-associated protein/Nuclear erythroid factor-related factor-2/antioxidant response element). Moreover, some sensitizers that bind cysteine residues, also directly activate the Keap1-Nrf2-ARE pathway (Natsch 2010),(Martin et al. 2011).

As a consequence of this recognition of contact sensitizers as danger signals, there is activation of intracellular signalling pathways and nuclear transcription factors, like p38 MAPKinases (mitogen-activating protein kinases) and NF-κB (nuclear factor of transcription κB) (Matos et al. 2005a),(Neves et al. 2011). Dependent on the P2X7 or Nrf-2 activation of the NLRP3 (NOD leucine-rich-containing family, pyrin domain-3) inflammasome and caspase-1, cytokines like IL-1β and IL-18 are cleaved, become activated and are secreted (Martin et al. 2011),(Honda et al. 2013), (Fig. B.11).

Contact sensitizers activate these multiple, and sometimes redundant, pathways in keratinocytes and skin DC, but also other skin cells, namely the fibroblasts, endothelial cells and mast cells.

In this whole process several cytokines and chemokines are secreted in the skin, like type 1 IFN, IL-1β, TNF-α, IL-6, IL-10, IL-12, IL-22, IL-23, CCL2/MCP-1 (monocyte chemotactic protein-1), IL-8/CXCL8, CXCL1, CXCL2, CXCL9, interferon induced protein of 10 KDa (IP-10/CXCL10) (Martin et al. 2011),(Rustemeyer et al. 2011),(Honda et al. 2013) (Fig. B.11).

During the sensitization process, DC are stimulated by cytokines and chemokines liberated from the keratinocytes or directly by the hapten, using pathways similar to those referred above, namely p38 MAPK, Keap-Nrf2-ARE, the inflammasome and caspase-1. DC become potent antigen presenting cells, i.e. they increase the expression of HLA molecules at the surface, some combined with the antigen, and express different costimulatory molecules, like CD54/ICAM-1, CD40, CD83, CD80/86, that facilitate the cross-talk with T cells. Also, DC produce new cytokines to influence the T cell response and change their morphology, becoming veiled and highly interdigitating cells, which facilitates contacts with many T cells (Kimber et al. 2011),(Rustemeyer et al. 2011).

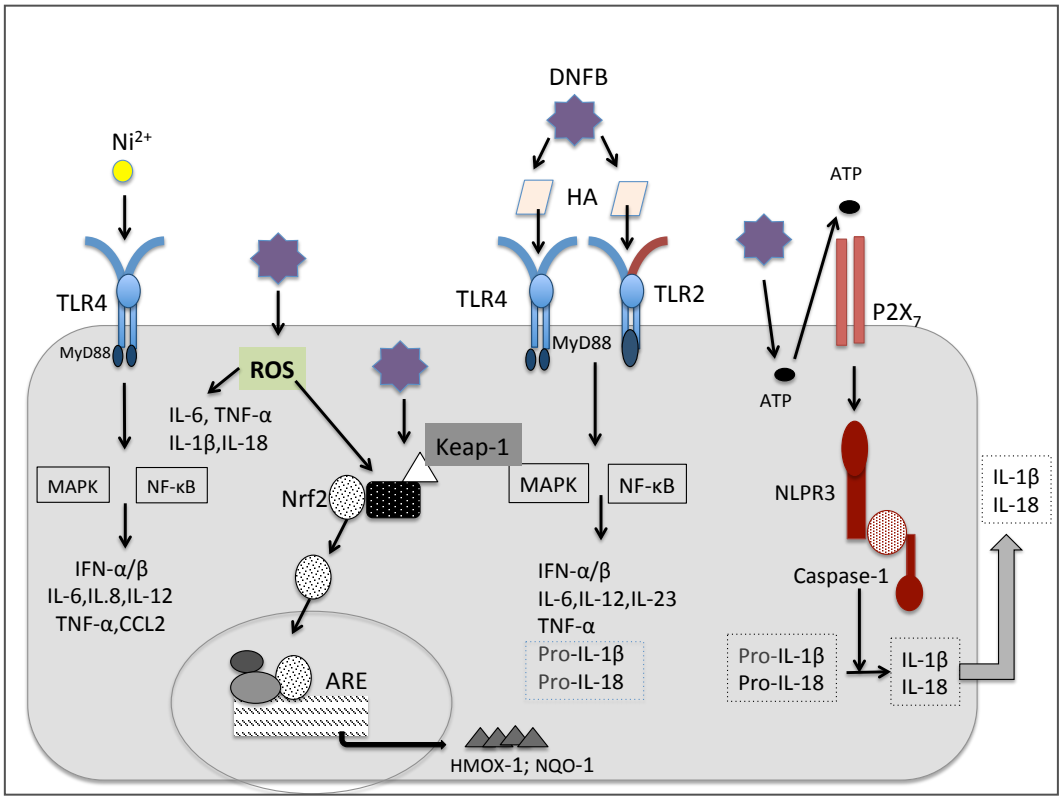


Figure B.11 – Schematic presentation of cell signalling pathways activated in keratinocytes and/or dendritic cells during the innate immune response to contact allergens, simplified and adapted from SF Martin et al. (2011). Nickel interacts with TLR4 and, through MyD88, it directly activates MAPKinases and NF-κB and the related pro-inflammatory cascade of cytokines and chemokines (IFN-type I, TNF-α, IL-6, IL-8, IL-12 and CCL2. Dinitrofluorbenzene (DNFB) induces reactive oxygen species (ROS) that activate the Nrf2/KEAP-1 pathway. Nrf2 is translocated to the nucleus where it activates the antioxidant response element (ARE) and the cell detoxifying enzymes hemeoxygenase-1 (HMOX-1) and NADPH quinone oxireductase-1 (NQO-1). Moreover, DNFB can induce hyaluronic acid fragments that are recognized as danger associated molecular patterns (DAMP) and active TLR4/TLR2. Additionally, DNFB induces adenosine triphosphate (ATP) formation, which activates the purinergic receptor P2X7 and the inflammasome NLRP3. Caspase 1 is sequentially activated and cleaves the inactive pro-IL-1β and pro-IL-18, allowing secretion of these cytokines to the extracellular fluid in their active form (Martin et al. 2011).

Moreover, upon the action of IL-1β, IL-18 and TNF-α, Langerhans cells reduce surface expression of E-cadherin, therefore loosening their attachments to neighbouring keratinocytes. They also express new adhesion molecules and chemokine receptors, namely CCR4, CCR7 and CXCR4, and produce metalloproteinases, like MMP-9, that will facilitate crossing of the dermal-epidermal junction and their migration, through the dermis, in their way to the regional lymph node (Basketter and Maxwell 2007),(Neves et al. 2008),(Martin et al. 2011),(Neves et al. 2011),(Kimber et al. 2011),(Martin 2012),(Honda et al. 2013). CCR7 is particularly important, as it guides the mature DC though the lymphatic vessels to the paracortical area of the lymph node, where naïve T cells, expressing also CCR7, are to be found (Rustemeyer et al. 2011) (Fig. B.12).

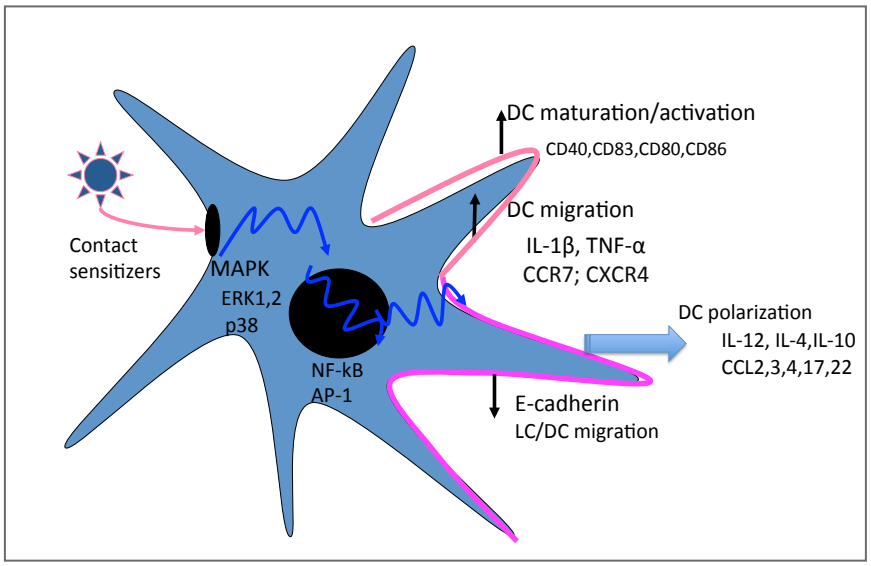


Figure B.12 – Activation of dendritic cells by contact sensitizers. These chemicals use different intracellular signalling pathways, namely MAPKinases, and nuclear transcription factors, as NF-κB and AP-1, and induce the expression of DC maturation markers like CD40, CD83, CD80 and CD86, which are important to communicate with T cells. Moreover, secreted cytokines (IL-1β and TNF-α) enhance DC migration out of the epidermis, and there is a modification of chemokine receptors that facilitates the way of DC through the dermis to the skin-draining lymph nodes (CCR7 and CXCR4). Langerhans cells also loose E-cadherin that keeps these cells attached to keratinocytes. Moreover, cytokines produced by DC may have an important effect on T cell polarization, with IL-12 facilitating a Th1 response, whereas IL-4 and 10 promotes a Th2 response, and IL-6 and IL-23 favours a Th17 profile.

In the draining lymph node, DC carrying the processed haptenated proteins may find naïve T-cells with the T cell receptor (TCR) that recognizes the culprit antigen combined with the HLA molecule. Antigen bound to HLA-class I cells will be recognized by $CD8^+$ T cells, whereas antigen complexed with HLA class II binds to $CD4^+$ T cells.

With an adequate number of adhesion molecules, namely intercellular adhesion molecule-1 (ICAM-1/CD54), DC establish contacts with many T cells through lymphocyte function antigen (LFA-1) until finding one with a compatible TCR. If, apart from interaction between TCR and the HLA molecule with the antigen, there are also costimulatory molecules both in the DC and in the T cell (CD80-86/CD28; CD40/CD40L) the immunological synapse will be completed (Fig. B.13).

In an adequate ambience of cytokines produced by the DC and neighbouring cells, naïve T-cells will be activated. They will produce high levels of IL-2, the classical T cell growth factor, and express high levels of the complete receptor for IL-2 (IL-2R), which, upon autocrine activation, will induce vigorous proliferation of the T cell clone. These T cells will develop into sensitized $CD8^+$ and $CD4^+$ specific effector and memory T cells. Some cells will recirculate mainly through peripheral organs, whereas recirculation of memory cells mainly within lymph nodes will be crucial for keeping central T cell memory (Rustemeyer et al. 2011).

In the absence of costimulatory signals and in the presence of a HLA/TCR stimulation of low intensity, regulatory T cells (Treg) producing IL-10 may preferentially develop. If the antigenic stimulus is very strong but there is no further costimulation, the T cell may undergo apoptosis or become anergic, that is, unable to be further activated in the presence of this sensitizer.

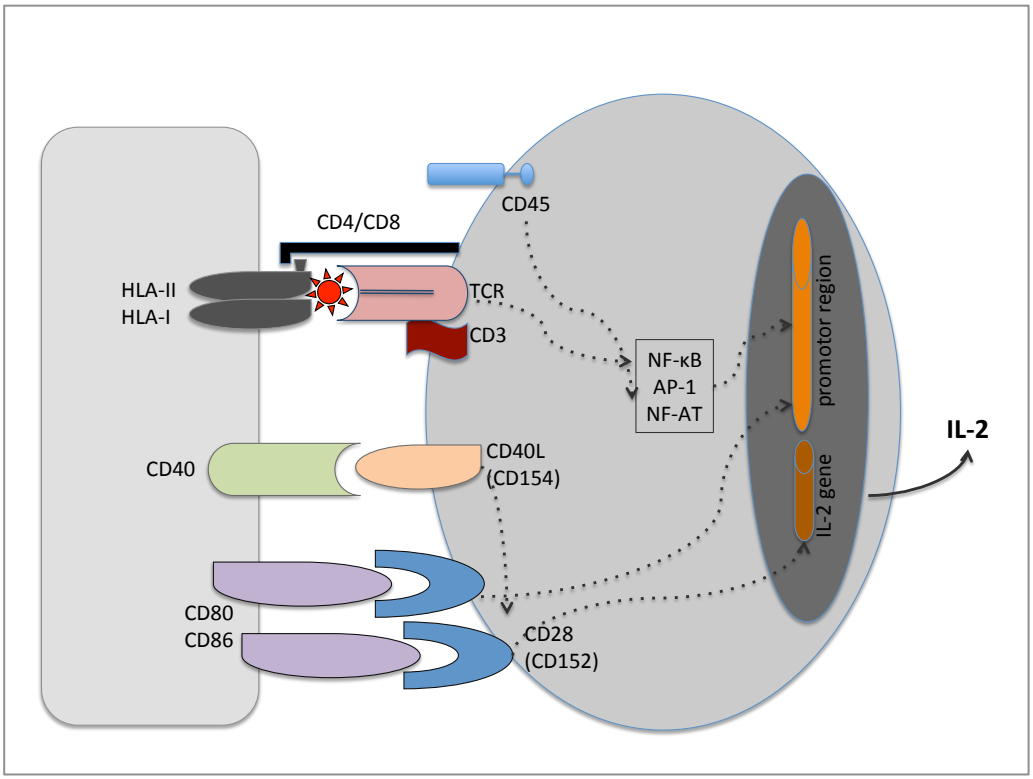


Figure B.13 – Dendritic cell (DC)/T cell interaction at the lymph node. The DC (left) carrying the Ag bound to HLA molecules binds the naïve T cell (right) whose TCR recognizes the combination HLA/Ag. The CD8 molecule binds a non-polymorphic area of class I molecules and, therefore, it is needed for the activation of $CD8^+$ T cells that recognize Ag bound to HLA-I. The CD4 molecule binds a related region in class II molecules and is necessary for $CD4^+$ T cell activation by Ag bound to class II molecules. After occupancy of the TCR there is signalling through CD3 complex, which is necessary for T cell activation. Nevertheless, other costimulatory signals delivered by DC (CD40 and CD80/CD86) are needed for complete T cell activation, proliferation and IL-2 production, i.e. to become sensitized T cells. The cytokine milieu may also be important to orient the T cell differentiation into the different sub phenotypes.

As the sensitization process occurs in a skin draining lymph node, most activated T cells will express the skin homing receptor, cutaneous leukocyte antigen (CLA), and also the chemokine receptors CCR10 or CCR4. In an ambience rich in IL-12, IL-18 and IFN-γ, that occurs after cutaneous exposure to most contact sensitizers, T cells will have mostly a Th1 phenotype. If high amounts of IL-23 and IL-6 are present, T cells will develop mainly into Th17

cells, whereas in an ambience rich in IL-4 and IL-10, which is induced by most respiratory sensitizers and some contact allergens, activated T cells will exhibit mainly a Th2 profile (Honda et al. 2013).

This T cell skewing depends, not only on the cytokine ambience during the sensitization process, but also on other aspects, namely on the nature, dose and type of exposure to the sensitizer and neuroendocrine factors from the host. The DC residing in the skin, which represent the interface between the innate and adaptive immune response in this process, are of extreme importance in the orchestration of the sensitization process and the future response.

Initial data suggested that Langerhans cells were the efficient antigen presenting cells for ACD, but more recent data suggest that they are not required. It has been suggested that Langerhans cells deliver mostly regulatory signals, whereas Langerin^{+} dermal DC, that reach the lymph node first, are the most efficient antigen presenting cells to generate T effector and memory T cells (Kimber et al. 2011),(Udey 2012),(Honda et al. 2013).

B.4.1.2. The effector phase

Once an individual becomes sensitized, the number of allergen-specific T cells increases significantly, representing up to $1{:}10^{4}$ of circulating T cells (Rustemeyer et al. 2011). Memory T cells, especially CLA^{+} T cells that recirculate through normal skin (about 200 T cells/h/cm2) will randomly enter the skin. If they encounter their specific antigen that was applied on the skin, *in loco* specific T cell proliferation induced by mature DCs combined with the antigen will occur (Honda et al. 2013). Therefore, during this effector phase of ACD, the skin will be enriched in allergen specific T cells (10-100 fold higher than in the blood), but most T cells that infiltrate the skin during ACD are not antigen-specific.

Some antigen-driven T cell specific migration may also occur, but most T cells are recruited in a non antigen specific way, attracted to the skin by a previous non-specific inflammatory stimulus, namely by CCL-2/MCP-1, the earliest chemokine detected in the skin after patch test application in sensitized individuals (Goebeler et al. 2001).

During the eliciting phase of ACD, the innate immune reaction induced by the sensitizer is of extreme importance to initiate the non-specific "irritant" inflammation that drives the specific adaptive response. Cytokines, chemokines and nitric oxide (NO) produced by keratinocytes, DC, fibroblasts, mast cells, monocytes and endothelial cells, all together, induce dermal capillary vasodilation and enhance the expression of adhesion molecules in endothelial cells, namely the E-selectin (CD62E), that attract memory CLA^+ T cells. Other cytokines and chemokines produced directly on exposure to the sensitizer or secondary to the inflammatory response, like IL-1β, TNF-α, CCL2/MCP-1, CCL5/RANTES, CCL20 and CCL22/MDC (macrophage-derived chemokine) will also attract other inflammatory cells.

Interferon induced protein of 10 KDa (IP-10/CXCL10) and CXCL9, together with CCL2 and CCL5, attract mostly Th1 cells, respectively carrying the receptors CXCR3 or CCR5. The chemokines CCL20 and CCL27/CTACK (cutaneous T-cell attracting chemokine) attract preferentially $CCR6^+$ and $CCR10^+$ T cells, including Th1 and, particularly, Th17 and Th22. Cells with a Th2 profile, rich in the chemokine receptor CCR4, are mostly attracted by CCL17/TARC (Thymus and activation-regulated chemokine). IL-8/CXCL8, produced in response to IL-17 and IL-22, will further attract neutrophils that may also be found in some lesions of ACD. Moreover, as shown in animal studies, INF-γ and IL-17 producing $CD8^+$ T cells attract TNF-α and iNOS (inducible nitric oxide synthase) rich monocytes, which also collaborate in skin inflammation (Chong et al. 2014). The relative predominance of these subphenotypes of effector T cells in the area of contact with the sensitizer may be responsible for clinical and histologic variations in the presentation of ACD.

The T cells, and also some other cells infiltrating the skin in ACD, produce cytokines (INF-γ, IL-4, IL-17, IL-22) that induce dermal inflammation and edema. Exudation progresses to the epidermis, inducing spongiosis and disruption of interkeratinocyte adhesion molecules to form vesicles. Moreover, T cells migrate to the epidermis (exocytosis) where specific effector T cells, associated with recruited NK and NKT cells, also have a cytotoxic effect on keratinocytes and Langerhans cells carrying the antigen (Rustemeyer et al. 2011),(Peiser et al. 2012). In this process, IL-17 makes keratinocytes particularly sensitive to the killing by Th1 cells (Pennino et al. 2010).

The ulterior infiltration of regulatory $CD4^+$ T cells (Treg) producing IL-10 will dampen the inflammatory response of ACD (Vocanson et al. 2009). Apoptosis of Th1 specific T cells which express high levels of Fas-ligand, and the preferential production of IL-10 during the late phase of stimulation of skin cells by the antigen may also contribute to the downregulation of the ACD response (Rustemeyer et al. 2011).

B.4.2. From pathophysiology of allergic contact dermatitis to delayed cutaneous adverse drug reactions

Like in ACD from topical chemicals, clinical and experimental data confirm the drug specificity and the involvement of delayed type hypersensitivity mechanisms dependent on drug specific T cells in delayed CADR from systemic drug (Roujeau 2005),(Pichler 2007),(Pirmohamed et al. 2011):

- the exanthema of the CADR usually begins within 7-21 days of drug use in the first episode, but it occurs earlier (1-2 days) after drug reintroduction (Friedmann 2003) and positive oral rechallenge can occur with lower doses (Lammintausta and KorteKangas-Savolainen 2005a), suggesting a specific reactivity;

- the culprit drug often elicits specific positive patch or intracutaneous tests with delayed readings (Barbaud et al. 2001a),(Lammintausta and KorteKangas-Savolainen 2005b),(Barbaud 2009),(Gonçalo and Bruynzeel 2011);

- in most delayed CADR, peripheral mononuclear cells isolated from the blood (PBMC) contain T lymphocytes that, *in vitro,* are specifically activated, proliferate or produce cytokines/chemokines on exposure to very small amounts of the culprit drug (Ebo et al. 2011),(Rozières et al. 2009a);

- for many years after resolution of the CADR, peripheral blood mononuclear cells (PBMC) keep the capacity to be specifically activated by the culprit drug, suggesting a long lasting memory (Fu et al. 2012);

- drug specific T cells and T cell clones have been isolated from the blood, and also from the skin, during the acute episode or, later, from positive patch tests (Yawalkar et al. 2000),(Kuechler et al. 2004);

- drug reactive T cells have been documented in the blood, with a frequency varying from 1:250 to $1:10^4$ of T cells (Beeler et al. 2006),(Fu et al. 2012), which is similar to the percentage of T cells reactive to contact sensitizers found in the peripheral circulation (up to $1:10^4$) (Rustemeyer et al. 2011).

T cell memory and effector T cells that recognize systemic drugs are definitively involved in nonimmediate CADR. In order to generate these reactive and mostly specific T cells in sufficient number, and by analogy with ACD, naïve T cells have to be stimulated by appropriate antigen presenting cells and in the proper ambience of cytokines/chemokines and costimulatory signals. This is considered necessary for a naïve T cell to become sensitized, proliferate and generate memory and effector T cells that will disseminate all over our body and generate the CADR upon further drug exposure. Nevertheless, there are many gaps in our knowledge of the complex mechanisms that lead to this state and, a direct inference from the knowledge of the pathophysiology of ACD, although very important for the understanding

of CADR, does not answer all the questions. There are some significant differences between the two reaction patterns, ACD and CADR, which do not allow us to fully complete the gaps in our knowledge.

In ACD, the chemical encounters the immune system through the skin-related immune and non-immune cells (keratinocytes, skin dendritic cells, fibroblasts and local-draining lymph nodes), whereas in CADR the first encounters of the drug with the immune system are variable and mostly unknown. In the blood circulation, in the liver, spleen or peripheral lymph nodes, many relevant immune cells may detect the drug, when it is administered intravenously. Upon oral drug intake, immune cells in the in the digestive mucosa, particularly in the gut, may also have the first encounter with this new chemical. And, in all routes of administration, the drug may reach the skin immune cells. The metabolic pathways and antigen presenting cells in these tissues are very different among them and different from the skin epidermal Langerhans cells and other skin DC or other dermal and epidermal resident cells.

In CADR, the immune status of the patient at the moment of drug exposure (concomitant drugs, infection, immunosuppression or immuno-inflammatory disease) may have significantly more influence than in ACD, where the local immune status of the skin during exposure to the contact sensitizer may be more relevant.

Drugs are pharmacologically active substances that may stimulate several cell receptors, including pharmacologic and immunologic active receptors and, consequently, influence the immune reaction, which would not be expected from contact sensitizers. Nevertheless, it has been increasingly shown that some contact sensitizers also directly activate immune cells, as is the example of nickel and related metals that stimulate DC through TLR-4 (Rachmawati et al. 2013).

In ACD, the nature, dose, type of exposure to the antigen and the consequent skin cytokine ambience, influenced by local metabolic, immune and

neuroendocrine factors, seem to dictate the type and strength of effector response. In CADR there are many other variables that influence the outcome, namely the drug and its pharmacologic activity, its dose and via of administration, individual genetics, concomitant diseases and concomitant drugs that may influence both antigen formation, its presentation to the immune system and the type of effector function.

We do not understand why a similar drug, under different situations, even in the same individual, induces different patterns of CADR (maculopapular exanthema, AGEP, SJS/TEN) that are presumably dependent on different T cell subphenotypes (Hausmann et al. 2012). This may be analogous to ACD that, occasionally, may also present under different clinical patterns (eczematous, pustular, lichenoid, erythema-multiforme-like, granulomatous or lymphomatoid), very probably dependent on the predominant effector T cell phenotype involved in the effector phase.

B.5. The sensitization phase in cutaneous adverse drug reactions

As reported in ACD, an induction or sensitization phase must also occur in CADR. Excluding some exceptional circumstances, the individual does not usually react with a specific immune response on the first contact with the drug. He has to develop a sufficient quantity of drug reactive T cells to be able to generate a specific effector phase.

Drugs that induce both delayed CADR and ACD, for instance corticosteroids, behave as other contact sensitizers in which concerns sensitization (Baeck et al. 2011),(Baeck et al. 2013). Therefore, we may assume that, also by systemic route, professional antigen presenting cells are involved and drugs may use pathways similar to contact sensitizers to be presented to the immune system.

Nevertheless, we have to be aware that, in CADR, drugs may suffer considerable metabolism, in the skin and elsewhere in the body, possibly giving rise to several haptens/antigens, which may be responsible for different forms of specific immune recognition for each drug.

Cells involved in antigen presentation of drugs may be others than the skin DC, as the drugs meet circulating B cells and other antigen presenting cells in many other organs, like the liver, spleen and gut.

Apart from behaving as an hatpen, drugs can be recognized by the immune system by altering the self-peptide repertoire on HLA molecules or they may have the additional possibility of a direct pharmacologic effect of the drug on relevant pharmacologic or immunologic receptors on antigen presenting or effector cells (*p-i* concept) (Karlin and Phillips 2014).

There are often general concomitant factors, apart from the drug (chronic inflammatory or infectious disease or other drugs), which influence drug sensitization and/or the elicitation of the CADR.

Genetic susceptibility markers, particularly HLA genotypes, have a strong influence on sensitization to some drugs, although, for the moment, this is not so apparent in ACD.

The capacity of the drug to stimulate the innate skin immune system, widely studied for contact sensitizers, may also be an important step in wakening the immune system to develop the adaptive immune response (Bellón and Blanca 2011).

Altogether, these aspects may confer special aspects to the induction or sensitization phase in CADR, but they are still far from being completely understood.

B.5.1. Antigens and haptens in drug hypersensitivity

As contact sensitizers, drugs are not usually recognized by the immune system as immunogens or complete antigens, e.g. they do not directly induce a specific immune response by themselves. They usually behave as haptens and need to combine with proteins/peptides to become antigenic and be capable of inducing an immune response.

Moreover, drugs are often prohaptens, i.e. a chemical formed as an intermediate or final product of drug metabolism or drug bio activation is usually the true hapten recognized by the immune system (Rozières et al. 2009b). Main drug metabolism is usually accomplished by hepatocytes but skin cells, including keratinocytes and DCs, also have very rich enzymatic machinery. Skin cells may be involved in the oxidation and formation of intermediate oxidative products that react with local proteins and form the sensitizing epitopes, as has been shown for sulfamethoxazole and carbamazepine (Merk 2009),(Sanderson et al. 2007),(Elsheikh et al. 2010).

Keratinocytes possess many cytochrome P-450 (CYP) isoenzymes, some common to the liver CYP enzymes, others exclusive to keratinocytes or hepatocytes. During biotransformation of xenobiotics, including drugs, the skin and liver enzymes may generate similar or different reactive metabolites and sensitizing chemicals in their tissues (Merk 2009). In the case distinct sensitizing metabolites from the same drug are formed and only one has sensitized the individual, we may assume the effector reaction may differently affect the skin and the liver. On the other hand, if the same sensitizing metabolite is formed both in the skin and the liver, the adverse reaction may similarly affect the organs that harbour the same sensitizing epitope.

Drugs that induce photoallergy suffer previous photoactivation. Ultraviolet (UV) energy transforms the drug, either into stable photoproducts or into reactive metabolites, which combine with proteins in order to become a complete antigen for T cell recognition. For instance, ketoprofen upon UV irradiation generates chemicals with a reactive benzophenone moiety that bind

aminoacids and generate specific reactive T cells (Sugiura et al. 2002),(Imai et al. 2005). This explains cross-reactions between ketoprofen and benzophenone UV-filters, octocrylene, fenofibrate and some of the arylpropionic NSAID that have a similar benzophenone structure (Devleeschouwer et al. 2008),(EMCPPTS Taskforce et al. 2012),(Gonçalo et al. 2013). New photoproducts can also be the hapten, as in photoallergy from piroxicam. This other NSAID is degraded by UVA into a photoproduct chemically and antigenically similar to the thiosalicylate moiety of the contact allergen thiomersal. Therefore, in individuals with previous contact allergy to thiomersal, topical or systemic exposure to piroxicam and sun exposure induces photoallergy (Gonçalo et al. 1992),(Gonçalo 1998).

As referred, the reactive drugs, their oxidative intermediates or final metabolites have to bind peptides to become antigenic. In most cases they establish covalent bonds with nucleophilic aminoacids (mainly cysteine or lysine) in soluble or cellular peptides, including albumin and MHC molecules (Park et al. 2007),(Sanderson et al. 2007).

Protein binding has been studied a long time ago for penicillin G and ampicillin and, more recently, also for flucloxacillin and piperacillin. Betalactam antibiotics usually suffer a nucleophilic attack, open the betalactam ring and form the penicilloyl group that binds lysine residues of proteins, in a time- and dose-dependent way (Weltzien and Padovan 1998),(Megherbi et al. 2009),(Whitaker et al. 2011). Hydrolysed and cyclized piperacillin haptens bind to one or several lysine residues of albumin and can be found in the circulation of patients exposed to this antibiotic, particularly those with cystic fibrosis (Whitaker et al. 2011). Piperacillin or flucloxacillin adducts with albumin are one of the possible sensitizers, are they are able to stimulate drug specific T cells, *in vitro* (Martin et al. 2010),(Whitaker et al. 2011). Although binding with lysine is predominant, some penicillin derivatives, like penicillenic acid, can also bind sulfhydryl groups of cysteine, therefore originating different antigenic epitopes (Weltzien and Padovan 1998).

One of the main sensitizing metabolites of sulfamethoxazole, nitroso-sulfamethoxazole, binds covalently to several thiol groups of soluble or cellular proteins (cysteine), including HLA molecules, or to thiol groups of glutathione (Park et al. 2007).

For each drug, as shown for sulfamethoxazole and penicillins, more than one epitope may have the potential to sensitize the individual and induce the CADR, which may have important implications for cross-reactions.

Interestingly, the epitopes needed for the sensitization process may be different from those that reactivate the memory T cells. In the particular case of nevirapine, the bioactive metabolite is needed for sensitizing naïve T cells, but memory T cells also recognize the parent drug (Merk 2009).

Apart from the different combination with peptides to be recognized by the TCR, the hapten mechanism, drugs can use this and other different pathways to be shown to the immune system (namely altered self-peptide repertoire and allorecognition). The antiretroviral abacavir, which has been widely studied, can be presented to the immune system and recognized through several of these pathways. In most cases, abacavir needs no drug metabolism and binds directly to a serine residue at position 116 in the F pocket of the antigenic groove of the HLA-B*57:01 molecule, localized either at the extra- or intracellular level. Specific $CD8^+$ T cells will then directly recognize the combination of the HLA molecule with abacavir, therefore a modified self-peptide. In an alternative process, abacavir, as an hapten, is combined with a protein and transferred to the endoplasmic reticulum in a TAP dependent way, to anchor exclusively the amino acid sequence in the antigen-binding pocket of the HLA-B*57:01 (Bharadway et al. 2012),(Yun et al. 2012a),(Chessman et al. 2008),(Bell et al. 2013). Still, in another possible pathway, the specific, although sometimes transient, binding of abacavir to the HLA molecules alters the chemistry and topography of the peptide binding groove. Upon these changes, different self-peptides can occupy the antigenic groove of HLA-B*57:01 or the self-peptides that regularly occupy the HLA groove will alter their binding sites

and change their final conformation, so that the HLA+new/modified self-peptide will be able to elicit an allo-reactive response by $CD8^+$ T cells (Bharadway et al. 2012),(Karlin and Phillips 2014).

A single amino acid replacement in the relevant spot of the HLA antigenic groove is enough to prevent the efficient association between abacavir and the HLA molecule and, therefore, hinders T cell recognition and sensitization. Cells carrying the very similar HLA-B*57:02, HLA-B*57:03 or HLA-B*58:01 do not bind abacavir efficiently and are not recognized or killed by abacavir specific T cells (Chessman et al. 2008),(Bell et al. 2013).

Recently it has been shown that, in some CADR, the drug combined with HLA or the modified HLA molecules can be "miss-recognized" as a viral peptide. Similarly to organ transplantation, where T cell responses against some relevant sequences of herpes virus that have previously infected the individual, may be cross-reactive with heterologous HLA-I molecules expressed in the donor organ, explaining an allogeneic $CD8^+$ T cell response and organ rejection, in CADR, a similar allogeneic $CD8^+$ response can develop to autologous HLA molecules modified by the drug. Interestingly these allogeneic T cell reactions do not need antigen processing and presentation and are organ-specific, as often occurs in CADR (Pavlos et al. 2014).

Therefore, and as a summary, the immune system can specifically recognize the drug and/or a metabolite bound to proteins/peptides (hapten hypothesis) or, eventually, a protein of the "self" (like a HLA molecule) modified by the reactive drugs or metabolites or, still, the drug can modify the way of binding of self-peptides to the HLA groove and, consequently alter the presentation of the usual tolerant self-peptides (Fig. B.14). Still, the drug combined with HLA may be recognized as a cross-reactive peptide (viral peptide) to which the individual was previously immunized (Karlin and Phillips 2014),(Pavlos et al. 2014).

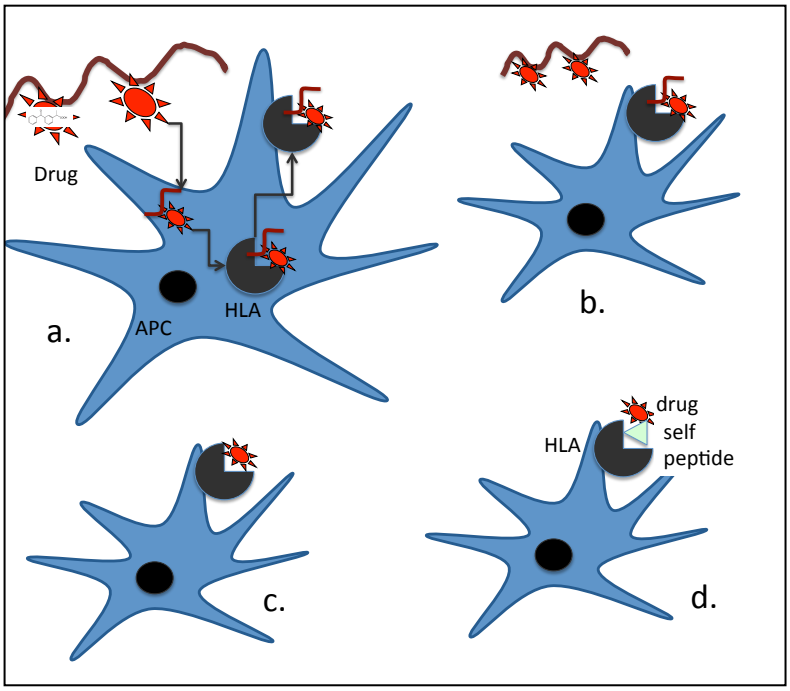


Figure B.14 – Drugs, behaving mainly as haptens, can use different pathways to be presented to the immune system. The drug or a metabolite combined with a peptide can suffer endocytosis by the antigen presenting cell, combine with HLA molecules in the endoplasmic reticulum and the complex HLA + peptide + drug is transported to the cell surface for T cell recognition (a). In some cases the drug/metabolite anchored to the peptide, or the drug itself, binds directly the antigen cleft of the HLA molecule, with no need for previous antigen processing. This modifies the HLA molecule that is then recognized by the TCR (b,c). Still, in other cases, the binding of the drug to the HLA molecule can change the peptides of the self that regularly occupy that particular HLA antigenic groove or the drug modifies their binding sites so that they become recognized by a TCR (d).

B.5.2. Antigen processing and presentation in drug hypersensitivity

As with contact sensitizers, it might be expected that drugs need professional antigen presenting cells to be processed, before being presented to naïve T cells.

At present, no data has shown which DC really participate in antigen presentation for nonimmediate CADR, neither in which lymph nodes does the

cross-talk between DC and naïve T cells occur in order to generate memory and effector cells. As skin-homing CLA^+ drug specific T cells are involved in CADR, it is expected that antigen presentation also occurs in skin draining lymph nodes.

Drugs administered orally or intravenously reach the skin where they can undergo metabolism in keratinocytes, DCs or other resident cells. If they are transformed here into the reactive hapten, they can also be processed by the epidermal and dermal DC and then presented to T cells in the skin-draining lymph nodes. This might explain the frequency of the skin involvement in the delayed immune mediated adverse drug reactions and the capacity of the skin to reproduce the reaction when the drug is applied in a cutaneous patch test (Barbaud et al. 2001a),(Barbaud et al. 2013a). The crucial importance of the skin DC in the antigen presentation could also justify the similar behaviour of drugs and contact sensitizers in the dendritic-like cell models, *in vitro* (Megherbi et al. 2009).

Circulating antigen presenting cells, e.g. DC, peripheral blood mononuclear cells (PBMC) and B cells may also participate in antigen presentation, as well as tissue DC from organs where the drug is concentrated or metabolised, namely the liver.

PBMC isolated from patients with delayed CADR, which contain many cells with antigen presenting capacity, can activate and induce proliferation of specific T cell clones *in vitro,* in the presence of the offending drug, but little is known about their capacity to sensitize naïve T cells.

Plasmocitoid dendritic cells (pDC), mainly involved in viral recognition, may also have an important participation in drug presentation or may be involved in the proliferation of viral related T cells observed in many drug eruptions, (Bellón and Blanca 2011), particularly in DRESS where pDC were shown to accumulate in the dermis (Sugita et al. 2010).

B-cells and NKT cells may also participate in the presentation and/or activation of effectors T cells, but little is known about their participation in delayed skin reactions. Contrary to DC, circulating B cells and monocytes isolated from

patients with delayed reactions from amoxicillin failed to activate T cells from these patients, *in vitro* (Rodriguez-Pena et al. 2006).

In some circumstances in CADR, drugs need antigen processing before they combine with HLA molecules for antigen presentation, whereas in other cases the drug, or the haptenated peptide, bind directly the HLA molecule without previous processing (Fig. B.14). As shown for abacavir, carbamazepine and allopurinol, when the drug directly binds the HLA molecule, altering the self-peptide repertoire of the HLA-molecule, and induces a response that reminds the allo-reactive T cell response in transplantation rejection, antigen processing and presentation may not be necessary (Karlin and Phillips 2014),(Yun et al. 2014).

In vitro studies have shown that, in patients with cotrimoxazole-induced maculopapular exanthema, some clones recognize the chemical combined directly with the HLA groove, even in fixed antigen presenting cells, whereas others need viable antigen presenting cells that are able to process the antigen before presenting it (Nassif et al. 2004a),(Pichler et al. 2006), (Naisbitt 2004).

In some cases the drug binds only transiently with peptides within in the HLA groove, as shown with carbamazepine, lamotrigine, several fluorquinolones and allopurinol (Pichler 2002),(Gerber and Pichler 2007),(Yun et al. 2014), whereas in some circumstances these, and other drugs, establish a long lasting covalent binding with the DC, at least *in vitro*. For instance, sulfamethoxazole metabolites, formed *in vitro* within the antigen presenting cells, are still bound to these cells in culture after many days (Elsheikh et al. 2010). It is possible that, also *in vivo,* antigen presentation proceeds for several days, using drug antigenic epitopes tightly bound to HLA molecules. Such a long lasting binding might explain why, in some cases, the skin reaction progresses long after drug withdrawal. Moreover, if these drug antigenic epitopes are also firmly bound to target cells, they may continue to be an available target for effector cells.

B.5.3. Drug recognition by specific immune receptors

Drugs can be specifically recognized by antibodies, namely by IgE fixed on mast cells and on basophils, in immediate hypersensitivity (Fig. B.1), or by IgG or IgM fixed on cells or in soluble immune complexes, respectively, in type II or type III hypersensitivity reactions. Nevertheless, there is no documentation of relevant antibodies recognizing the drug in delayed CADR.

In nonimmediate CADR, T cells recognize the drug, but there are several forms of drug recognition. Usually T cells recognize the drug, as haptens, combined with the HLA molecule, through their specific receptor, the T-cell receptor (TCR). It is mostly a αβ TCR with the relevant sequence of amino acids in the hypervariable region that, as for classical antigens, recognize the complex (drug+peptide+HLA) in a MHC restricted manner, with drugs combined with class I being recognized by $CD8^+$ cells and those combined with class II by $CD4^+$ T cells. Nevertheless, for an ideal recognition and effective T cell activation, the number of HLA molecules present on antigen presenting cells, particularly those carrying the drug/hapten, and the avidity of the binding between the HLA and TCR, may influence the outcome of this cross-talk.

This form of hapten recognition has been shown for penicillins, cephalosporins, norfloxacin, sulfamethoxazole, abacavir, carbamazepine, lamotrigine, and phenobarbital (Chessman et al. 2008),(Rozières et al. 2009b). Nevertheless, each drug can combine and form distinct antigenic peptides. Therefore, different TCR and, consequently, different T cell clones can recognize the drug or its metabolites, which is compatible with a polyclonal or oligoclonal T cell response, usually observed in CADR. Nevertheless, the affinity of the different T cell clones may be highly variable.

This variability of the TCR response, partially dependent on the antigenic epitope recognized by the TCR, has important implications on the pattern of T

cell cross-reactivity *in vitro* and, consequently, also influences cross-reactivity at a clinical level.

In patients with maculopapular exanthema induced by cotrimoxazole, T cell clones or T cell lines isolated from the blood recognize the chemical in a MHC restriction manner, but some recognize only sulfamethoxazole, only its intermediate metabolites, like hydroxylamine sulfamethoxazole or nitroso sulfamethoxazole, or both. T cells that recognize sulfamethoxazole also recognize other anti-infectious sulfonamides with a same conformational structure (Nassif et al. 2004a), whereas clones that recognize nitroso-sulfamethoxazole have a more restricted cross-reactive pattern (Naisbitt 2004).

Cross-reactivity, *in vitro,* at the T cell level, between carbamazepine and oxcarbamazepine is the rule, but in some patients T cells recognize only carbamazepine and patients tolerate the pro-drug oxcarbamazepine (Ko et al. 2011). In our personal experience, patients with DRESS from oxcarbamazepine have positive patch tests to carbamazepine, confirming the frequent cross reactivity detected at T cell level (unpublished data).

In delayed hypersensitivity from penicillins, the recognized epitope is either the betalactam ring or its the side chain. In the first case, there are cross-reactions between different penicillins and, in the second hypothesis, cross-reactions between aminopenicillins and amino-cephalosporins, like cefaclor that shares the R1 side-chain with ampicillin (Romano et al. 2005), (Romano et al. 2013).

Although not studied at T cell level, the recognition of piroxicam by the immune system depends on the pattern of drug reaction and, consequently, cross-reactivity also depends on the clinical pattern of drug reaction. The thiosalicylate moiety, formed after UVA irradiation is responsible for photoallergy. As this photoproduct is exclusive for piroxicam, other oxicams like tenoxicam can safely be used in photoallergy. On the contrary, in fixed drug eruption the immune system recognizes an oxicam moiety common to tenoxicam and, therefore, almost all patients with fixed drug eruption to

piroxicam cross react with tenoxicam (Gonçalo et al. 1992),(Oliveira et al. 1999).

For some drugs, special amino acid sequences, important for drug recognition, have been identified in the hypervariable TCR region. Carbamazepine, when combined with HLA-B*15:02, is recognized by T cells mainly with the amino acid sequences Vβ-11-ISGSY. The presence of this TCR that recognized carbamazepine was observed in 84% of the HLA-B*15:02 positive Han Chinese who developed SJS/TEN. Also, on *in vitro* exposure to carbamazepine, PBMC from patients with SJS/TEN who carry such TCR suffer an oligoclonal proliferation, whereas HLA-B*15:02 positive patients who tolerate carbamazepine have no such no such oligoclonal T cell proliferation, which suggests the importance of the amino acid sequence in the TCR for the complete carbamazepine recognition and, consequently, for developing SJS/TEN (Ko et al. 2011),(Ko and Chen 2012). Therefore, both the presence of HLA-B*15:02 and the existence of T cells relevant TCR are necessary to develop this adverse reaction.

Requirement for this subpopulation of T cells with the Vβ-11-ISGSY region is not extensive for other patterns of drug eruptions induced by carbamazepine or for populations other than the Han Chinese (Phillips et al. 2011),(Roujeau et al. 2011). In European studies, almost all carbamazepine, as well as lamotrigine-reactive T cell clones generated from skin infiltrating and circulating cells during the CADR, carried the Vβ 5.1 chain and some recognized the drug on HLA-II matched antigen presenting cells. Also, data in HLA-B*15:02 positive Han Chinese suggested that Vβ-11 region of the TCR was important for developing SJS/TEN (Ko et al. 2011). These data suggested that, in these cases, carbamazepine and, also, lamotrigine, might also have a superantigen-like effect and, therefore, induce a more polyclonal T-cell proliferation (Poszeczynska-Guigné et al. 2005). Nevertheless, other TCR portion, distinct from the hypervariable region, which may have influence on drug recognition, has not been much more explored. Also, one of the most studied drugs in this

aspect, abacavir that is combined with HLA-B*57:01, is recognized by TCR with no predilection for any Vβ region (Chessman et al. 2008),(Bell et al. 2013).

B.5.4. Pharmacologic drug effect: the *p-i* concept

Another possible mechanism of drug interaction with the immune system has been proposed by Werner Pichler, the '**p**harmacological interaction with **i**mmune receptors', the *p-i* concept (Pichler et al. 2006).

In pharmacological receptors, the drug often binds transiently by its steric and electronic characteristics, activates the receptor and produces the pharmacologic effect. Similarly to this model, in CADR, it is supposed that the drug can establish reversible interactions with different receptors in T cells. If the drug binds the hypervariable, antigen specific portion of the TCR, it may induce a T cell proliferation with the same TCR repertoire, therefore in a very drug specific way. On the other hand, if the drug interacts with another relevant and active immune T cell receptor, independent of the TCR, e.g. CD3 or CD28, the drug can induce T cell activation and proliferation, independent of the TCR/antigen specificity. In both situations, a T cell population will expand, either more oligoclonal or more polyclonal, with more or less drug affinity. This T cell population will, nevertheless, be capable of generating the skin reaction (Pichler et al. 2006),(Pichler et al. 2011) (Fig. B.15).

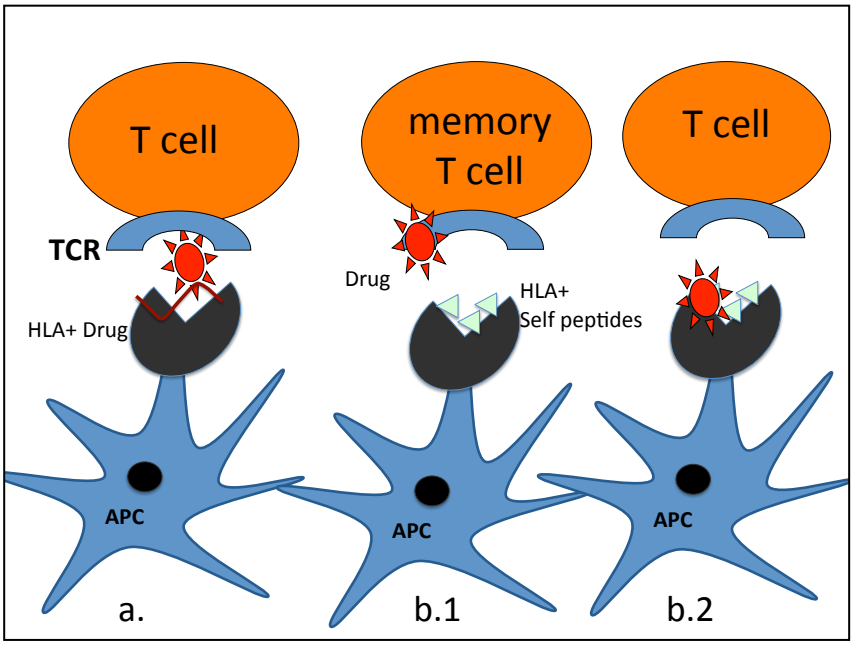


Figure B.15 – Drug presentation to T cells. The hapten hypothesis versus the p-i concept, adapted from Hausmann *et al.* (Hausmann et al. 2012). In the hapten hypothesis (a) the drug/metabolite covalently bound to the HLA molecule in the antigen presenting cell is specifically recognized by the hypervariable portions of the T cell receptor (TCR). T cells resulting from the proliferation of this T cell will share the same TCR. In the pharmacologic – interaction (***p-i***) concept, the drug binds transiently to the TCR **(*p-i*-TCR)** (b.1), or to the HLA molecule **(*p-i*-HLA)** (b.2), and is able to maintain contact between antigen presenting cells and the T cells, and, therefore, activates several T cells expressing the functional receptors, although not specifically the hypervariable portion of the TCR. This presupposes a pre-activation of the T cell with expression of many functional receptors. Moreover, proliferating T cells may have different TCR, therefore the response may be more polyclonal.

The *p-i* concept was proposed based on some particular findings of delayed CADRs.

1. Some drugs, like radiologic contrast media, induce T cell mediated skin eruptions on the first contact and usually within a few hours, therefore with no time for the sensitization process (Gerber and Pichler 2007).

2. Some drugs bind transiently and do not establish long lasting covalent bounds with MHC molecules in antigen presenting cells. In agreement, in some *in vitro* experiments, washing the cells to eliminate the free/non cell-bound

drug completely abrogated T cell activation, as shown for abacavir, carbamazepine and allopurinol/oxypurinol (Yun et al. 2014).

3. Some T cell clones, *in vitro,* are activated with no need for a viable active antigen processing/presenting cell and other T cell clones exhibit no MHC restriction.

4. Still, both *in vitro* and *in vivo*, more T cell clones are activated than those that exhibit a drug specific TCR (Pichler et al. 2006).

Therefore, there is the possibility that some drugs transiently bind proteins of the MHC complex or, particularly, the TCR, even outside the antigenic groove of these molecules, and maintain enough contact between the MHC of the antigen presenting cell and the TCR of memory T cells to induce their activation and expansion. These represent, respectively, the *p-i*-HLA or the *p-i*-TCR concept.

Moreover, drugs might stimulate other relevant immune receptors pre-expressed in T cells, receptors eventually activated by other factors, and the drug will, in this way, induce T cell proliferation. An argument in favour of this hypothesis within the *p-i* concept is based on the fact that a concomitant viral infection (EBV, HHV-6, HIV disease) or an immuno-inflammatory disease enhances CADR. In these settings, T cells express more adhesion and costimulatory molecules and, therefore, are already in a pre-activated state with a lower threshold for activation and proliferation, i.e. they exhibit functional receptors that might be directly activated by the drug, bypassing the regular antigen processing and presentation (Pichler 2002),(Gerber and Pichler 2007),(Pichler et al. 2006).

Although these arguments support this theory, the *p-i* concept has not been fully demonstrated experimentally and it is important to reinforce that this effect does not exclude that the drug can, concomitantly, activate the immune system in a more specific way, as a regular hapten or prohapten (Pichler et al. 2006), and that both mechanisms can potentiate each other.

B.5.5. Drug dependent innate immune reaction

By analogy with contact sensitizers and ACD, during the initial process in CADR and before the activation of the adaptive immune reaction, the drug or its reactive metabolites may induce non-specific "danger signals" or "alarmins" (Blázquez et al. 2011), some of which have been detected in the skin or in the circulation, particularly in severe CADR.

As contact sensitizers, drugs may activate cellular receptors regularly used by common pathogens, modify the oxy-redox state of the cell or interfere with components of the tissue matrix, that will be recognized as danger signals by "non-immune" guardians of our body (keratinocytes, fibroblasts, endothelial cells, mast cells). These skin resident cells or other relevant cells in the body, sensing the presence of the drug as a danger signal, will secrete cytokines and chemokines that awaken the sentinels of the adaptive immune system, the dendritic cells. In this ambience of "danger", the DC acquire full capacity for antigen presentation and T cell stimulation. In the absence of this innate response, antigen presenting cells may keep in their immature state and, instead of activating effector and memory T cells, they rather induce a tolerogenic response (Chaves et al. 2010), which fortunately seems to happen most of the time.

In drug eruptions, circulating and tissue NK cells, belonging to the innate immune response, may also play a part in the first encounters of the drug with the immune system. They secret cytokines (IFN-γ) or liberate their granules that either enhance the activity of dendritic antigen presenting cells or kill the immature DC, hindering their antigen presentation to tolerogenic cells (Chaves et al. 2010).

For contact sensitizers, *in vitro* tests have studied their capacity to activate an innate immune response in cultures of keratinocyte, DC or mixed cultures. Some very few studies have addressed the capacity of systemic drugs to activate

dendritic cells *in vitro* and most of these studies used DC isolated from the peripheral blood of patients or control individuals.

Human DC developed *in vitro* from PBMC of healthy volunteers and patients with sulfamethoxazole-induced maculopapular exanthema were directly stimulated by the drug, increasing CD40 expression (Sanderson et al. 2007),(Elsheikh et al. 2010).

Using the THP-1 cell line or other monocyte derived DC, with the same methodology as for contact sensitizers, some studies showed direct activation of these dendritic-like cells by penicillins (penicillin G, V and ampicillin) with upregulation of ICAM-1/CD54 expression, secretion of IL-1β and activation of anti-oxidant response element genes (ARE) (Nukada et al. 2011),(Sebastian et al. 2012). Sulfamethoxazole showed no effect in this *in vitro* model, explained by the lack of sufficient cytochrome P metabolizing enzymes in these cells to convert sulfamethoxazole into its reactive metabolite (Sebastian et al. 2012). A Japanese group using the THP-1 cell line in a study with hepatotoxic drugs that can also induce CADR, albendazole, amiodarone and terbinafine, also observed that these drugs upregulate the maturation markers CD86 and CD54 in this DC-like model and increase the secretion of IL-8 and TNF-α (Endo et al. 2012).

Therefore, as contact sensitizers, the systemic drug, by itself or its reactive metabolites, may also have an inherent capacity to cause some degree of xenoinflammation *in vitro*, enhance the activity of antigen presenting cells and, consequently, promote its own presentation to the immune system and facilitate the generation of drug specific T cells (Martin et al. 2011).

Interestingly, the Spanish group of Malaga observed immune activation of both dendritic and NK cells isolated from the circulating blood, but exclusively from allergic, not from amoxicillin tolerant patients. Upon *in vitro* exposure of these blood derived immature dendritic and NK cells to amoxicillin or heparins, DC upregulate the surface expression of CD86 and HLA-DR and, in parallel, increase the capacity to stimulate T cell proliferation (Rodriguez-Pena et al. 2006),(Blázquez et al. 2011). On amoxicillin exposure, NK cells also upregulate

cytotoxic molecules (perforin and granzyme B) and increase cytokine production (IFN-γ), but, again, this occurs exclusively in allergic patients (Chaves et al. 2010). This may suggest individual differences in the innate recognition of chemicals as danger signals or, eventually, also a previous activation of innate receptors (PRR) that enhance the response. Moreover, cell populations used in these studies may not be completely free of drug specific memory cells, which could influence the DC or NK response.

B.5.6. Concomitant predisposing factors in drug hypersensitivity

Concurrent factors at the time of drug exposure are of extreme importance for the development of the CADR, as shown in several clinical settings. Concomitant use of aminopenicillins and allopurinol seems to represent a risk factor for developing CADR (Pérez et al. 2001); patients with cystic fibrosis, either due to perturbed immune status with frequent infections or extensive exposure to antibiotics, have a much higher risk of cutaneous adverse reactions to antibiotics (Whitaker et al. 2011),(El-Ghaiesh et al. 2012); systemic lupus erythematosus or HIV infected patients are more susceptible to CADR, particularly from sulphonamides (Allanore and Roujeau 2007),(Naisbitt 2004); during EBV or CMV infection, antibiotics, particularly aminopenicillins, induce a maculopapular exanthema in a high proportion of patients, but only a few become sensitized and develop a skin rash on drug re-exposure without the concomitant infection (Renn et al. 2002); and, during the last decades, attention has been drawn to the association of DRESS/DIHS with reactivation of human herpes virus (HHV) of the Betaherpesvirinae subfamily, namely HHV type 6 (HHV-6), the agent of the infantile exanthema, roseola infantum (Uno et al. 2014) and, more recently, also with HHV-7, CMV and EBV (Descamps et al. 1997),(Kano et al. 2004),(Shiohara et al. 2007),(Descamps and Ranger-Rogez 2014).

The higher frequency of CADR in these settings can be explained either according to the p-i concept or to the hapten hypothesis, as they increase the formation of drug protein adducts, increase the expression of inflammatory receptors on immune cells, create a cytokine rich ambience, eventually a "cytokine storm" and, therefore, influence the immune status of the patient, which may enhance either the sensitization and/or the effector phase of the CADR.

Concomitant exposure to other reactive chemicals or drugs that stimulate antigen presenting cells act as "danger" signals that alert the adaptive immune system to go into a state of pre-activation and monocyte/macrophages or DC become increasingly capable of presenting the drug to T cells (Bellón and Blanca 2011),(Bellón et al. 2010).

During infectious and chronic immuno-inflammatory diseases (Still's disease, systemic lupus erythematosus), bacterial toxins, viral proteins or nucleic acids are recognized as PAMP (pathogen associated molecular patterns) and endogenous cellular peptides liberated from dying/dead cells during the inflammatory process are recognized as DAMP (death/damage associated molecular patterns). Both DAMP and PAMP stimulate membrane associated Toll-like or cytosolic NOD-like receptors in keratinocytes or other epithelial cells and in DC or other antigen presenting cells and, consequently, these cells will liberate cytokines that alert the adaptive immune system. In these settings, most drugs, even those that lack an intrinsic capacity to activate the innate immune response, can be more easily presented to the immune system and stimulate the specific immune response.

Reinforcing this aspect, Lavergne *et al.* showed that antigen presenting cells are more active in their function under *in vitro* stressful conditions that mimic a concomitant exposure to a microbial infection. In their experiments, pre-exposing dendritic-like cell cultures (THP-1, HL60 and PMBC) to several external stresses (bacterial LPS, viral products, pro-inflammatory cytokines and increased temperature) before adding sulfamethoxazole, significantly increased the formation of protein adducts with sulfamethoxazole and its reactive

metabolites in DC (Lavergne et al. 2009) and, consequently, the capacity of DC to stimulate T cells. A similar situation occurs in cystic fibrosis, whose patients frequently exposed to infections and antibiotics, have significantly more circulating albumin/antibiotic adducts than other patients exposed to the same antibiotics (Whitaker et al. 2011),(El-Ghaiesh et al. 2012).

According to the *p-i* concept, apart from the more active and efficient drug presentation to the immune system, a large population of T cells involved in the previous immune and inflammatory process will have a lower threshold for activation. Due to cytokine ambience and the expression of a significantly higher number of relevant immune receptors on these memory T cells, drugs may "pharmacologically" stimulate these receptors and induce proliferation of effector T cells responsible for the CADR (Gerber and Pichler 2007).

B.5.7. Genetic susceptibility for cutaneous adverse drug reactions

Pharmacogenetics and pharmacogenomics, recently enriched with new research tools, like genome-wide association studies (GWAS), are very active areas of research in CADR. Significant relations between some genetic traits and delayed CADR to particular drugs have already been proven and new ones are being evaluated.

These studies establishing the relation of some CADR with particular characteristics of drug metabolizing pathways, immune and inflammatory genes, TCR repertoire and, particularly, with certain HLA haplotypes has been extremely important to understand pathophysiologic mechanisms in CADR but, particularly, to define risks, adopt preventive measures and, therefore, reduce adverse drug effects (Wei et al. 2012c),(Pirmohamed 2012),(Karlin and Phillips 2014).

Inborn errors of drug metabolizing enzymes are mainly responsible for toxic effects due to overdosing as, for example, hematologic or hepatic toxicity in

thiopurine S-methyltransferase deficient patients exposed to azathioprine or mercaptopurine (Wei et al. 2012c).

olymorphisms within drug metabolizing and detoxification enzymes, namely in cytochrome P_{450}, epoxide hydrolase or glutathione transferase, may produce different intermediate reactive (or non-reactive) drug metabolites or distinct amounts of the culprit metabolite involved in the hypersensitivity reaction (Lavergne et al. 2008), as initially suggested for anticonvulsants and DRESS (Husain et al. 2013). Recently, Wei Chung and co-workers, using GWAS studies, have shown a strong association with SNP detected in chromosome 10 and severe CADR from phenytoin. These SNP code for CYP2C9 mutations with a deficient activity in the metabolism of phenytoin and these patients exhibit unusually high serum drug levels that decrease very slowly after stopping the drug, which may be responsible for more severe reactions.

Polymorphisms in immune and inflammatory response pathways may increase the risk and severity of some drug reactions in a nondrug-specific way. Predisposition to produce higher levels of soluble FasL and polymorphisms in the TNF-promoter region have been associated with severe bullous reactions as SJS/TEN (Lerch and Pichler 2004),(Lan et al. 2006) and recent studies suggest that mutations in IL-36RN are very probably associated with AGEP (Navarini et al. 2013),(Sugiura et al. 2014).

Although all these genetic aspects may influence the susceptibility or severity of CADR, the strongest genetic risk factors for CADR demonstrated thus far are related to HLA haplotypes and some specific drugs. At present, this is a very active area of research within the field of pharmacogenomics, with attempts to define precise risk association for many drugs and adopt preventive measures, which has already very significantly reduced severe CADR, namely from abacavir and carbamazepine.

HLA association is related with the capacity of the drug to combine or insert into the HLA groove of antigen presenting or target cells. Interestingly the binding capacity is not equal for all drugs or its metabolites and, somehow unexpectedly, this HLA relation is not only drug-dependent but, sometimes, also dependent on the population and the phenotype of the drug eruption (Phillips and Mallal 2009b),(Phillips et al. 2011),(Yun et al. 2012a),(Karlin and Phillips 2014).

An isolated report from Italy, dating back from 1997, showed an association between fixed drug eruption from feprazone and HLA-B22, but apparently this has never been replicated (Pellicano et al. 1997).

The first strong recognized association was published by Chung *et al*, in 2004, for HLA-B*15:02 and carbamazepine-induced SJS/TEN (2500-fold increased risk), in Han Chinese, in Taiwan (Chung et al. 2004),(Hung et al. 2005b). This was later reproduced in many other studies (Yang et al. 2007),(Chen et al. 2011),(Wei et al. 2012a),(Hashizume, 2012) and this association with HLA-B*15:02 and SJS/TEN from carbamazepine was extended to other Asian populations, except the Japanese, and also to other aromatic anticonvulsants as oxcarbamazepine, phenytoin and lamotrigine, although with a lower risk (Aihara 2011). This association is not extensive to other patterns of severe CADR from these anticonvulsants, namely to DRESS, and does not occur in Europeans. A more recent study found an association between HLA-A*31:01 and carbamazepine-induced DRESS/DIHS, SJS/TEN and maculopapular exanthema in European and, also, in Japanese populations, although with a much lower risk (McCormack et al. 2011),(Ozeki et al. 2011),(Aihara 2011),(Lichtenfels et al. 2014). The association with HLA-A*31:01 is also relevant for children with carbamazepine induced CADR (Amstutz et al. 2014a), but is not extensive to other aromatic anticonvulsants, namely lamotrigine or phenytoin (McCormack et al. 2012).

A strong association was also detected for HLA-B*58:01 and severe drug reactions from allopurinol, both SJS/TEN and DRESS/DIHS. This association was first suggested in southern Chinese, as early as 1989, by SH Chan and T

Tan (Zineh et al. 2011) and more widely explored after 2005, when SI Hung et al. published 51 cases (21 SJS/TEN and 30 DRESS) in Han Chinese, all of them carrying this haplotype, whereas this allele was present in only 15% of allopurinol tolerant individuals (Hung et al. 2005a). This strong association is observed in Han Chinese from China (Cao et al. 2012), Thailand (Tassaneeyakul et al. 2009), Hong Kong (Chiu et al. 2012), Korea (Jung et al. 2011),(Kang et al. 2011) and those living in Australia (Lee et al. 2012b), with an OR (Odds Ratio) varying from 34.00 (in smaller samples) to 348.88 (mean 96.60; 95% confidence interval (CI) 24.4-381.00; $p < 0.001$) in SJS/TEN and also in DRESS (Somkrua et al. 2011). This association is not so strong among other Asian and Australian patients, namely in Japan where only 36-40% are HLA-B*58:01 positive (Lee et al. 2012b),(Kaniwa et al. 2008). In Europe, studies were performed only in SJS/TEN and HLA-B*58:01 was present only in 19 out of 31 patients (61%), but only in 15 out of 27 of European ancestry (55%) (Lonjou et al. 2008). A stronger association, similar to the Han Chinese was, nevertheless, found in a study in the Italian island of Sardinia, where all 18 SJS/TEN cases were positive for this allele (Atzori et al. 2012).

Thus far, the strongest and most established association worldwide is between HLA-B*57:01 and a potentially fatal hypersensitivity syndrome from abacavir. This particular form of DRESS/DIHS develops in a short time interval, with no eosinophilia but a significant hepatic and gut involvement and is confirmed by patch tests (Phillips et al. 2002),(Phillips et al. 2005),(Phillips and Mallal 2009a), (Phillips and Mallal 2009b). HLA-B*57:01 has a positive predictive value of 55% and, the absence of this haplotype, has a negative predictive value near 100% (Phillips and Mallal 2009b),(Phillips et al. 2011). Worldwide recommendations for a mandatory search for this haplotype, before initiating therapy, have practically eliminated abacavir hypersensitivity from clinical practice.

In all these studies, the stronger associations occurred with HLA-class I molecules, particularly with the more diverse HLA-B locus. As antigens bound to HLA-class I molecules are recognized by $CD8^+$ T cells, this can justify why most drug specific T cells found to be involved in delayed drug hypersensitivity

are $CD8^+$ cells, namely for abacavir and carbamazepine (Chessman et al. 2008), (Phillips and Mallal 2009b).

In contrast, for the antiretroviral drug nevirapine, the strongest but not definite association is with the HLA class II antigen, HLA-DRB1*01:01. Interestingly, this drug induces a more heterogeneous pattern of hypersensitivity reactions that are apparently mediated by drug specific $CD4^+$ T cells and are highly dependent on the patient's circulating $CD4^+$ T cell count (Phillips and Mallal 2009b). Also, recently, in a European patient with DRESS from carbamazepine, apart from HLA-A*31:01 that was important for the activation of $CD8^+$ T cell clones, HLA-DRB1*04:04, that is in linkage disequilibrium with the HLA-A*31:01, was shown to be relevant for the activation of $CD4^+$ T cell clones isolated from the same patient (Lichtenfels et al. 2014).

Associations between HLA haplotypes and some drugs are very interesting but there are some aspects that seem difficult to explain, particularly in which concerns variability depending on the populations and CADR patterns studied.

Some HLA haplotypes predispose to a single pattern of drug reaction whereas others predispose to several clinical patterns induced by the same drug, namely HLA-B*15:02 is associated with SJS/TEN but not with DRESS from carbamazepine.

Moreover, it is not yet explained how ethnicity influences the strength of the risk, as for allopurinol, where HLA-B*58:01 confers a much higher risk for severe reactions in Han Chinese than in Europeans or in other Asian populations, namely the Japanese. Other genetic traits that concur with HLA as risk factors for CADR, namely in drug metabolism, or epigenetic modifications may, eventually, explain how ethnicity influences the strength of association with HLA.

In other cases, ethnicity may completely change the risk factor associated with a HLA genotype. HLA-B*15:02 is associated with a very high risk factor for carbamazepine-induced SJS/TEN in Han Chinese, whereas in Europeans and

Japanese, HLA-A*31:01 showed the higher risk, both for carbamazepine-induced SJS/TEN and DRESS (McCormack et al. 2011),(Ozeki et al. 2011).

Also, for very similar HLA genotypes, like HLA-B*57:01 and HLA-B*58:01 which belong to the HLA-B*17 serotype, the risk association is for two distinct drugs, respectively, abacavir and allopurinol. On the other hand, for the same drug carbamazepine, the risk of CADR is associated with structurally unrelated HLA molecules, namely HLA-B*15:02 for Chinese and HLA-A*31:01 for Europeans (McCormack et al. 2011),(Yun et al. 2012a).

Moreover, some individuals do not develop the reaction even though they have the proper genotype and are equally exposed. In this setting, a specific T cell receptor may be necessary for drug recognition, as shown for carbamazepine (Ko et al. 2011), previous sensitization to cross-reactive viral epitopes or concomitant factors (chronic inflammatory disease or active infection) may be important for developing hypersensitivity (Pavlos et al. 2014).

Apart from HLA, other still unknown particularities will certainly explain individual susceptibility and this may occur at a very initial step of the drug sensitizing process. Immature DC from patients with amoxicillin hypersensitivity, *in vitro,* are more responsive to the drug than controls (Rodriguez-Pena et al. 2006). For the moment, there is no explanation for such a different reaction from cells that apparently belong to the innate immunity and are supposed to have no memory, neither a very specific pattern of reactivity.

In certain situations, the genetic background is really a considerable risk of developing CADR, particularly certain HLA and severe drug eruptions. The identification of these HLA associations represents one of the first big steps in order to develop a more personalized Medicine, adapting individual genotypes to the prevention of adverse drug events. Maybe, in the future, this also

extends to the choice of the most efficient drugs, based on the individual genotype (Wei et al. 2012c).

B.6. Effector mechanisms in cutaneous adverse drug reactions

The effector phase of the immune mediated delayed drug reactions is complex, with particular aspects depending on the clinical phenotype of the CADR. In common, delayed CADR involve drug specific T cells, which confer specificity to the reaction, but a significant part of the skin aggression is certainly due to non drug-specific T cells recruited to the site of inflammation. Moreover, skin resident cells, particularly keratinocytes and DC, also participate in the effector phase, either as target cells or by secreting cytokines and chemokines that attract inflammatory cells. Other skin resident or recruited cells, like macrophages, NK and NKT cells, eosinophils, neutrophils, mast cells, fibroblasts and endothelial cells, also participate in the effector phase of the CADR.

The dose and chemical properties of the drug, the culprit antigenic epitope formed, the innate immune response generated, the cytokine ambience and type of antigen presenting cell activated upon drug exposure, will influence the T cells subsets that are activated and recruited to orchestrate the inflammatory skin reaction.

Individual patients' characteristics and underlying diseases or concomitant factors occurring during drug exposure also influence the final clinical outcome of the adverse reaction. Nonetheless, it is not yet known why, for patients exposed to the same drug, some develop maculopapular exanthema, others AGEP, some have a few localized bulla as in fixed drug eruption or widespread bullae with major skin detachment, as in SJS or TEN.

B.6.1. Drug specific effector T cells

Drug specific T cells have been isolated from the skin, during the acute reaction or from positive skin tests, and from the blood, during the CADR or many years after its resolution, even with no further drug exposure (Beeler et al. 2006).

During the acute episode, particularly in severe CADR, as DRESS, AGEP and SJS/TEN, drug specific T cells collected from the blood are particularly numerous and show signs of activation (Rozières et al. 2009b),(Beeler et al. 2006). Amoxicillin and sulfamethoxazole specific T cells represent up to 30-125 cells/10^6 PBMC, according to the evaluation by enzyme-linked immunospot (ELISpot) assay. This number is similar to the circulating nickel reactive T cells found in patients with ACD from this metal (Rozières et al. 2009a). In another study, using both ELISpot and LTT (lymphocyte transformation tests) but considering only CD4+ T cells, Beeler *et al.* observed that 1:10.000 up to 1:250 of all $CD4^+$ T cells isolated from the blood responded specifically to the responsible drug (Beeler et al. 2006).

In vitro, these cells can be stimulated and proliferate on exposure to small amounts of the culprit drugs, but, occasionally the response is dose-dependent, as shown for oxypurinol (Yun et al. 2014). Nevertheless, conditions needed to undergo T-cell activation are not always the same, namely in which concerns the requirement for environmental cytokines or antigen presenting cells. Some drug-specific T cells respond in the absence of professional antigen presenting cells, but most often they require antigen presentation in the context of autologous or heterologous professional and functional antigen presenting cells. Occasionally, fixed cells can also accomplish antigen presentation (Pichler et al. 2006). Also, a proper HLA molecule may be necessary for T cell stimulation, as shown for abacavir, carbamazepine and allopurinol (Lichtenfels et al. 2014),(Bell et al. 2013),(Yun et al. 2014).

Drug specific T cells, isolated from the skin or from the blood, are oligoclonal or polyclonal memory and effector T cells, with different phenotypes ($CD4^+$,

$CD8^+$, Th1, Th2, Th17, Tc1) and, also, different *in vitro* effector activity. They differ in their cytotoxic armamentarium (Fas-Ligand, perforin/granzyme B or granulysin) and cytokine profile (TNF-α, INF-γ, IL2, IL-5, IL-8/CXCL8, IL-17, IL-22). Most initial studies reported mainly type 1 and/or type 2 $CD4^+$ and $CD8^+$ T cells (Rozières et al. 2009b), but, recently, Th17 cells as well as $CD4^+$ T cells with regulatory activity (IL-10 and TGF-β production) were also shown to be involved (Pichler et al. 2011).

The phenotype diversity of isolated T cell subsets very probably correlates with their *in vivo* effector function and, therefore, with the type of skin aggression usually observed in the different patterns of CADR (Rozières et al. 2010),(Pichler et al. 2011). Moreover, the intensity of mediator release may also dictate the intensity of skin aggression. For instance, drug specific T cells isolated from patients with SJS/TEN and with maculopapular exanthema, both produce high levels of INF-γ and Fas Ligand (FasL) but these effector molecules are produced in much higher in SJS/TEN patients, which is in agreement with the most intense apoptotic skin reaction (Fu et al. 2012)

There is not one single T cell phenotype responsible for a pattern of CADR, although some phenotypes are mainly involved in some specific patterns of drug eruption. The relative proportion of the different reactive T cell subphenotypes involved and their active mediators, in conjunction with the response of resident and recruited cells, dictate the final clinical phenotype of the CADR.

Based on the T cell subtype involved mainly in each pattern of CADR, Werner Pichler subdivided type IV hypersensitivity reactions into four subgroups (IVa – IVd) (Lerch and Pichler 2004),(Pichler 2007).

Type IVa are reactions mediated predominantly by T-helper 1 cells, rich in IFN-γ, which activate monocytes and macrophages.

Type IVb reactions involve T cells producing mostly type 2 cytokines (IL-4 and IL-5), preferentially activating and recruiting eosinophils.

Type IVc include cytotoxic reactions mediated by $CD4^+$ and, particularly, by $CD8^+$ T cells. These cytotoxic T lymphocytes (CTLs), rich in perforin, granzyme B, granulysin and FasL, are mainly involved in bullous reactions.

Type IVd reactions are mediated by T cells that promote neutrophil inflammation through IL-8/CXCL8. Under the new subdivision of the T helper cell profiles, this category would probably fit mainly into the Th17 subtype (Kabashima et al. 2011),(Maddur et al. 2012). The prototype of Type IVd is AGEP, although these neutrophil recruiting T cells may also contribute to other drug eruptions (Pichler 2003),(Hausmann et al. 2012). Other cutaneous diseases rich in neutrophils, like psoriasis, acne, hidradenitis suppurativa, Beçhet's disease and some auto-inflammatory syndromes, may share common pathogenic pathways with AGEP (Keller et al. 2005),(Schlapbach et al. 2011a),(Lowes et al. 2013),(Thiboutot et al. 2014) (Fig. B.16).

This subclassification of type IV hypersensitivity is very interesting for understanding mechanisms involved in CADR, but also in other T cell mediated diseases, like the drug induced liver injury (DILI), chronic asthma, and the skin tuberculin test, as shown in figure B.16.

Generally, in CADR, drug specific circulating T cells exhibit high levels of CLA, CCR4 and CCR10, turning them highly prone for skin migration. CLA, the skin homing receptor, is recognized by E-selectin-rich skin endothelial cells. The chemokine receptors, CCR4 and CCR10, direct T cells towards related chemokines secreted by keratinocytes and other skin cells, namely, CCL17/TARC, CCL22/MDC and CCL27/CTACK (Tapia et al. 2007).

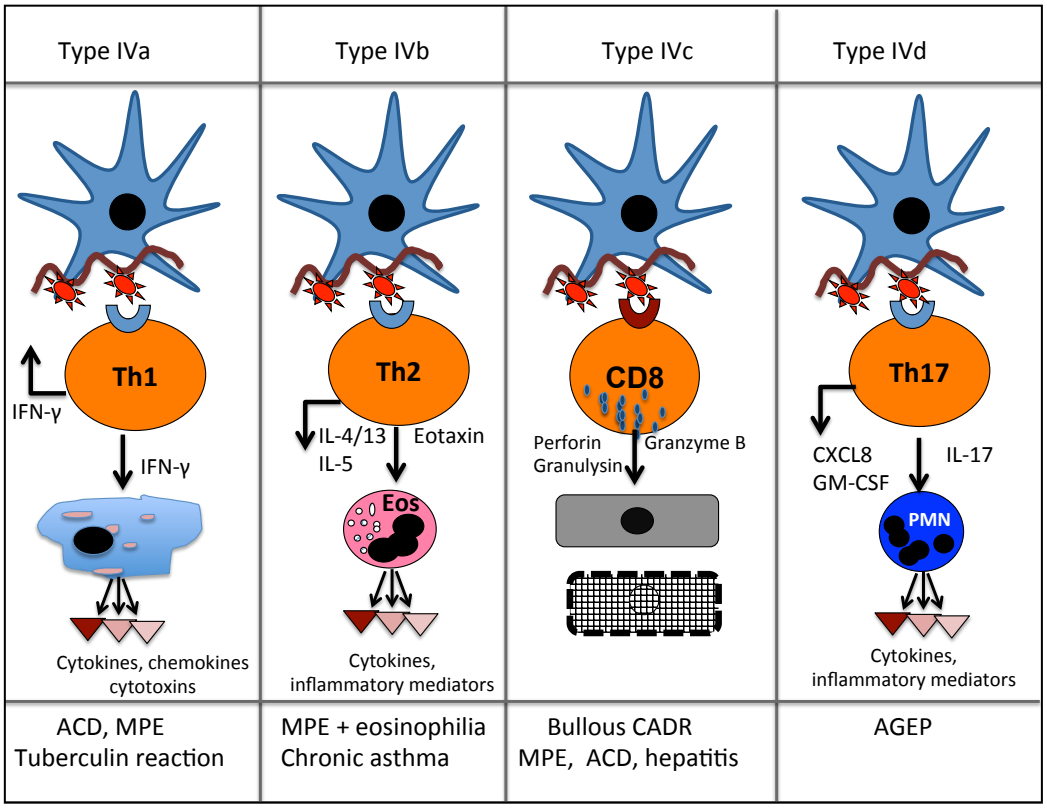


Figure B.16 – Representation of the different subtypes of delayed hypersensitivity reactions (type IVa – IVd) involved in CADR and related immune mediated reactions, adapted from publications by W. Picher (Hausmann et al. 2012). The antigen presented by dendritic cells (in blue) activates different subtypes of T cells. In type IVa, Th1 cells are activated and liberate IFN-γ that activates monocytes and macrophages to digest antigens but also to secrete many cytokines, chemokines and other cytotoxic factors. Type IVb reactions involve mostly type 2 cytokines, IL-4/13 and IL-5, which recruit and activate eosinophils (Eos), whose granules can induce cellular lesions. Type IVc include cytotoxic reactions mediated mainly by $CD8^+$ T cells or other cytotoxic T lymphocytes rich in perforin, granzyme B and granulysin. These granules induce apoptosis/necrosis of target cells combined with the antigen (in grey). Type IVd are reactions mediated by IL-17 secreting T cells that promote neutrophil inflammation (PMN) through IL-8/CXCL8.

B.6.1.1. $CD4^+$ T cells

Reflecting a Th1 predominance, drug specific $CD4^+$ T cells produce mainly INF-γ, but this cytokine is usually associated with TNF-α, IL-2 IL-4, IL-5 and

IL-13. INF-γ activates DC, monocytes and macrophages enhancing their function, namely the expression of HLA-II molecules involved in antigen presentation. Moreover, INF-γ has an effect on target cells, like the keratinocytes, that become activated, also express high levels of HLA-II and produce chemokines, like CXCL10.

The Th2 cytokine, IL-5, particularly involved in eosinophil activation is an important marker in many drug eruptions (Yawalkar et al. 2000),(Kuechler et al. 2004), and not only in DRESS, where blood and tissue eosinophilia is more significant.

Some drug specific CD4+ T cells also produce perforin and granulysin which are considered responsible for cytotoxicity against keratinocytes in several CADR, particularly in SJS/TEN (Porebski et al. 2013). This occurs particularly if, upon the action of IFN-γ, keratinocytes are induced to express HLA-II molecules on their surface (Yawalkar et al. 2000),(Pichler et al. 2002),(Kuechler et al. 2004),(Yawalkar 2005).

Th17 cells, and eventually Th22 cells, that produce IL-17A/F, IL-22 and IL-21 are involved in many drug eruptions, particularly those with a neutrophil predominance, as AGEP (Kabashima et al. 2011). IL-17, IL-22 and IL-21, acting on keratinocytes, facilitate an ICAM-1 dependent cytotoxic effect by T cells. Moreover, these cytokines induce keratinocyte production of IL-8/CXCL8, a potent neutrophil chemoattractant involved in AGEP (Pennino et al. 2010)(Kabashima et al. 2011),(Maddur et al. 2012),(Teraki et al. 2013).

Some drug specific $CD4^+$ cells that express the Foxp3 gene ($CD4^+CD25^+Foxp3^+$) behave as regulatory T cells (Treg). They produce IL-10 and TGF-β that downregulate the inflammatory T-cell reaction. Actually, some authors agree that most $CD4^+$ T cells that infiltrate the skin in CADR, as well as in ACD, participate mainly as regulatory T cells (Vocanson et al. 2009), whereas $CD8^+$ are the real effectors.

An impairment of the Treg function is usually associated with more intense CADR. Evidence of this impairment has been demonstrated particularly in SJS/TEN (Takahashi et al. 2009) and can explain the more frequent occurrence

of CADR in autoimmune disorders (Hashizume, 2012). On the other hand, when these Treg cells are more active, they can allow an extensive viral reactivation, which is supposed to contribute to skin and multiorgan lesions in DRESS (Ogawa et al. 2013).

B.6.1.2. $CD8^+$ T cells

At present, drug specific $CD8^+$ cells are considered the main memory effector T cells in most CADR (Rozières et al. 2009b),(Rozières et al. 2010). $CD8^+$ T cells infiltrate skin biopsies and $CD8^+$ T cell clones are generated *in vitro* by T cell stimulation with the drug. $CD8^+$ T cell involvement in CADR is also consistent with the main susceptibility factors being related to HLA-class I, as $CD8^+$ T cells recognize antigens bound to HLA class I molecules (Chessman et al., 2008). $CD8^+$ T cell participation is particularly evident for drugs that bind HLA-class I molecules, as abacavir, and for patterns of drug eruptions with prominent cytotoxicity and bullous lesions.

Nevertheless, like in ACD, we may have to be cautious with some of these results taken from biopsies taken from a single point in the evolution of CADR or from data driven mainly from animal studies, as these favoured the role of $CD8^+$ T cells whereas human studies showed the preferential involvement of $CD4^+$ T cells in ACD (Dyring-Andersen et al. 2013).

$CD8^+$ T cells produce high levels of IFN-γ and contain cytotoxic/cytolytic granules rich in perforin, granzyme B and granulysin. The granules are liberated when, in a close cell-to-cell contact, the TCR of the lymphocyte recognizes a modified self HLA-class I molecule in the target cell. Perforin opens “holes” in the cell membrane of the target cell, which allows the penetration of granzyme B that initiates apoptosis.

The cationic protein granulysin, with 2 isoforms (9- and 15-KDa), is liberated into the extracellular fluid, particularly after T cell activation, and is effective at a distance with no need for cell-to-cell contact (Chung et al. 2008),(Hashizume, 2012). The smaller 9-KDa isoform of granulysin penetrates intact cells membranes, causes ionic imbalance and mitochondrial damage, activates caspases and induces apoptosis, which is considered a very relevant mechanism for keratinocyte apoptosis in SJS/TEN (Abe et al. 2009),(Zawodniak et al. 2010),(Araujo et al. 2011),(Tohyama and Hashimoto 2012),(Chung and Hung 2012). The 15-KDa isoform has recently been recognized as an "alarmin" that activates other cells, namely DC and monocytes, by stimulating TLR-4/MyD88 pathway (Hashizume, 2012). This 15-KDa isoform of granulysin, is significantly increased in the blister fluid of TEN (300x compared to a blisters from burn injuries) and can be detected in the systemic circulation in TEN and, in lesser amounts, in other CADR (Chung et al. 2008),(Abe et al. 2009),(Saito et al. 2012).

$CD8^+$ T cells exert their cytotoxic effect against autologous resting keratinocytes that express HLA-class I molecules combined with the culprit drug. Actually, in CADR, granulysin and granzyme B rich cells accumulate near the epidermal basal cell layer, in skin areas where there is more vacuolar degeneration and higher $CD8^+$ T cell infiltration. These granulysin and granzyme B rich cells are particularly numerous in SJS/TEN and in fixed drug eruption, where there is a major degree of keratinocyte apoptosis (Pichler et al. 2011),(Schlapbach et al. 2011b).

Most $CD8^+$ T cells have a type 1 cytokine pattern. TNF-α and IFN-γ produced by drug specific T cells upregulate Fas (CD95) expression on the keratinocyte surface, stimulate keratinocyte iNOS and increase nitric oxide (NO), which, together, turn keratinocytes much more susceptible for apoptosis (Viard-Leveugle et al. 2013). Apoptosis is facilitated, particularly, on exposure to death receptor ligands produced by mononuclear cells, such as TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) and TNF-related weak apoptosis inducer (TWEAK) (Araujo et al. 2011),(Tohyama and Hashimoto 2012).

B.6.1.3. NK and NK/T cells

Cytotoxic granules, particularly granulysin, are not exclusive of $CD8^+$ T cells. NK cells and NK/T cells are also rich in these granules and they represent a significant number of granulysin rich cells in the skin infiltrate during the acute eruption, particularly in CADR with the highest levels of cytotoxicity. In TEN, a subset of NK cells ($CD3^-$, $NKp46^+$) represent up to 13% of lymphocytes and accumulate in the dermal/epidermal interface and blister fluid, whereas in maculopapular exanthema they represent less than 4% of infiltrating cells and are mainly detected in the dermis (Schlapbach et al. 2011b).

Even though these NK cells do not have a TCR to specifically recognize the drug, *in vitro*, they are stimulated in a drug specific manner to upregulate granulysin production (Schlapbach et al. 2011b).

Moreover, Morel and Bellón have shown that amoxicillin can combine with free amino groups within certain HLA molecules of target cells and these cells become vulnerable to the NK cytotoxic attack (Morel and Bellón 2007). In normal circumstances, cells of the self are protected from cytolysis by NK cells as long as they express a correct amount of HLA molecules that activate inhibitory NK cell receptors. When there is a change in the number of HLA molecules at the cell surface or when HLA is modified and becomes distinct form the self, as after combination with amoxicillin and other drugs, NK cells tend to destroy such cells. This process may be relevant in the effector response of delayed CADR and explain the increase of NK cells present in the biopsies of these cutaneous reactions.

B.6.2. Interaction between effector and target cells

The different subtypes of drug-specific effector cells orchestrate the reaction in consonance with resident skin cells, with the objective of eliminating cells

modified by the culprit drug. T cells induce a cytotoxic effect on cells carrying the drug or cause an inflammatory reaction that will, secondarily, eliminate cells bound to the drug.

The main targets of effector cells are the keratinocytes, which suffer apoptosis in many CADR, even when no bullous lesions are observed. Other resident cells, like DC, fibroblasts or endothelial cells combined with the relevant drug epitopes, can also be targets for T-cell mediated destruction. In some circumstances, innocent cells, e.g. cells not combined with the culprit drug, also suffer some non-specific aggression, particularly in SJS/TEN.

In CADR with simultaneous systemic involvement, namely with hepatotoxicity, similar aggressive mechanisms are expected to occur in target organs, but they are very probably mediated by effector T cells with different patterns of homing receptors.

Apart from drug specific T cells or NK/NKT cells that destroy targets combined with the culprit drug, many non-specific cells are attracted to the inflammatory scene and contribute to the final reaction. Some inflammatory cells are recruited by “danger signals” or alarmins” produced by skin cells, either as an innate immune response to the presence of the drug or as the response of keratinocytes to their initial aggression. Other cells are recruited secondarily by the cytokines and chemokines secreted by activated keratinocytes and lymphocytes.

The eosinophil, attracted by IL-5 and eotaxin, is frequently observed in the skin in CADR, and, sometimes, also in high numbers in the circulation, as in DRESS. The neutrophil is also observed in CADR lesions, but predominantly in AGEP. These granulocytes with the content of their granules (eosinophil cationic protein, major basic protein, myeloperoxidase, elastase) also contribute to skin aggression.

Endothelial cells of skin capillaries, that exhibit adhesion molecules and E-selectin, are extremely important in the selection and recruitment of effector

cells to the site of the CADR. Moreover, by combining some drug epitopes, they can recruit drug specific T cells or be the target of their aggression.

There are distinct mechanisms of target cell aggression, but this has been studied mainly for the keratinocyte. It depends on the effector cells and their aggressive machinery or on the cytokine ambience, as previously referred and summarized in Fig. B.17 and Table B.1. Accordingly, there are distinct clinical and histopathologic skin lesions in the various patterns of CADR.

Table B.1 – Brief summary of the **main subtypes of effector cells involved in delayed CADR**, their main transcription factors, how they are activated, the cytokines needed for their differentiation, cytokines and chemokines produced and their main target cell

T cell subtype (transcription factor)	$CD4^+$ Th1 (STAT4)	CD4+ Th2 (STAT6)	$CD4^+$ Th17 (STAT3)	CD4+25+ Treg (Foxp3)	$CD8^+$ cytotoxic	NK/NKT $CD16^+CD56^+$ $NKp46^+$
Activation	APC + HLA-class II molecules				APC+HLA-I	Non-self
Cytokine for activation	IL-12, IL-18	IL-4, IL-13	IL-23, IL-6 TGF- β	TGF-β IL-10, IL-2		
Main cytokine production Effector mediators	INF-γ IL-2 TNF-α	IL-5 IL-4 IL-13 IL-10	IL-17 IL-21 IL-22 (IL-8/CXCL8)	IL-10 TGF- β	Granzyme B Perforin Granulysin FasL/CD95L INF-γ IL-2 TNF-α	Granzyme B Perforin Granulysin
Recruited activated or target cells	Monocyte DC keratinocyte	Eosinophil	Neutrophil Keratinocyte		Keratinocyte	Keratinocyte

Th-Thelper cell; Treg – regulatory T cells; STAT-signal transducer and activator of transcription;
APC – antigen presenting cell; DC – dendritic cell; NK – natural killer cell; IL-interleukin;
TGF- β – transforming growth factor-β; TNF-α – tumor necrosis factor-α; CXCL – CXC chemokine;
INF-γ – interferon-γ

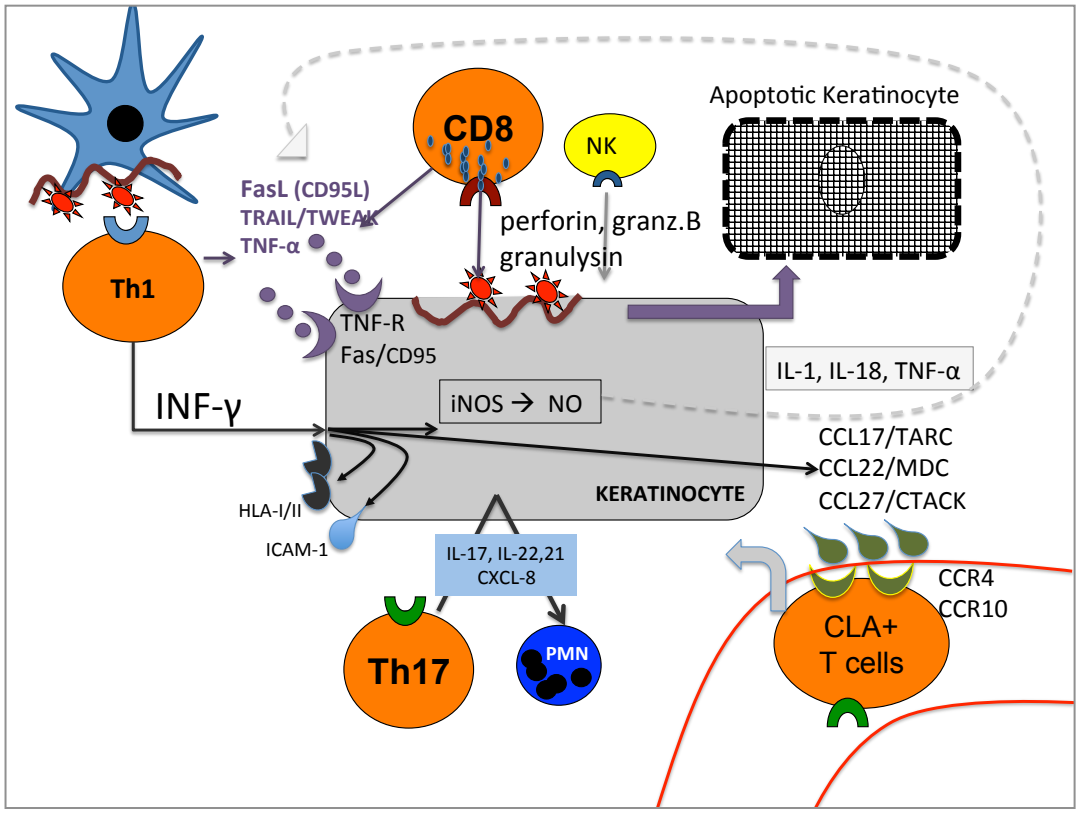


Figure B.17 – Simplified schematic presentation of the main cells involved in delayed CADR and their effect on keratinocytes. Briefly, the keratinocyte is the target for cytotoxicity and, as it suffers aggression, it secretes cytokines and chemokines that amplify the inflammatory loop and the killing of other keratinocytes. **1.** After recognizing the drug bound to HLA-I molecules on keratinocytes $CD8^+$ T cells, and also Natural killer (NK) cells, liberate their cytotoxic granules containing perforin, granzyme B and granulysin. **2.** Th1 cells, upon drug recognition on HLA-class II molecules, produce mainly IFN-γ that renders keratinocytes susceptible to apoptosis. **3.** Upon stimulation by IFN-γ and TNF-α, produced both by $CD8^+$ T cells and Th1 cells, keratinocytes upregulate the expression of HLA molecules and ICAM-1 at its surface, favouring contact with other cells. They also increase nitric oxide (NO) production by the inducible isoform of the enzyme NO synthase (iNOS) and upregulate the expression of death receptors that can be activated by Fas Ligand, TNF-α, TNF-related apoptosis inducing ligand (TRAIL) and TNF-related weak apoptosis inducer (TWEAK). **4.** As the keratinocytes suffer some damage, they secrete non-specific cytokines like IL-1, IL-8 and TNF-α that further amplify both keratinocyte apoptosis and the inflammatory loop. **5.** Moreover, chemokines secreted by keratinocytes (CCL17, CCL22 and CCL27) attract circulating CLA+ skin homing T cells expressing the corresponding chemokine receptors (CCR4 and CCR10). These T cells infiltrate the skin and may further contribute to inflammation. **6.** In CADR where Th17 cells are important, namely in AGEP, IL-17 and, eventually IL-22 and IL-21, activate the keratinocyte inducing the secretion of IL-8/CXCL8, a potent chemokine for neutrophils (PMN).

B.7. Effector mechanisms in specific cutaneous adverse drug reactions

In the different patterns of delayed CADR, although common effector mechanisms occur, there are some particularities in the phenotypes of participating effector cells, their cytokines and chemokines, and on the way they interact with resident and recruited cells to induce the particular CADR with a distinct clinical phenotype. Nevertheless, the existence of common pathways of skin aggression, referred above, may justify that overlapping patterns are occasionally observed in clinical practice.

B.7.1. Maculopapular exanthema

The effector mechanisms that initiate a maculopapular exanthema in a sensitized individual are not precisely known. It is supposed that drugs administered systemically reach the skin and combine with keratinocytes or skin DC. Eventually, a local innate immune inflammatory reaction occurs, with production of IL-1β, IL-6, IL-18, TNF-α, which attracts the first drug specific circulating cells to the skin. Upon finding the drug on DC or keratinocytes, T cells initiate their activation on site (Rozières et al. 2009a). Inflammatory products liberated by keratinocytes and other skin resident cells and/or by drug specific T cells will further recruit other circulating cells that cause skin inflammation.

In maculopapular exanthema the skin is infiltrated by skin homing CLA^+, $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells are observed mainly in the epidermis, where vacuolar or apoptotic keratinocytes occur, and $CD4^+$ are found mainly in the dermis, around blood vessels. Eosinophils and, occasionally, neutrophils, are also found in the dermis (Naim et al. 2011),(Seitz et al. 2013).

It is supposed that skin homing T cells adhere to cutaneous vessel walls that express higher amount of adhesion molecules, through the interaction of T cell

adhesion molecules, CLA and LFA-1, and its respectively ligands, namely E-selectin and ICAM-1, in endothelial cells. They are then attracted by chemokines secreted by keratinocyte and DC, like the CCL17/CCL22 and CCL27 that selectively recruit skin homing memory T cells expressing respectively, CCR4 and CCR10 (Naisbitt 2004),(Tapia et al. 2004),(Brönnimann and Yawalkar 2005),(Tapia et al. 2007).

Once activated in the skin, T cells secrete a heterogeneous profile of cytokines and chemokines, usually with a predominance of a type 1 cytokines (IFN-γ and TNF-α). They activate DC and keratinocytes increasing their expression of HLA class I and class II molecules. These molecules bind more drugs and present it further to T cells, or are the target for T-cell mediated cytotoxicity.

During the acute phase, both $CD4^+$ and $CD8^+$ T cells expressing perforin, granzyme B and granulysin exert a cytotoxic activity against keratinocytes. This is manifested by vacuolar degeneration, particularly near the basal layer, dyskeratosis in scattered cells in the upper epidermal layers (Yawalkar 2005),(Seitz et al. 2013) and formation of intraepidermal vesicles (Bircher 2012).

IL-5, a type 2 cytokine, along with the CCL11/eotaxin-1, is responsible for the recruitment and activation of eosinophils, considered a local and systemic hallmark of cutaneous maculopapular drug eruptions (Bellini et al. 2013). Nevertheless, the presence of eosinophils in the skin is not constant, even in maculopapular exanthema where a drug specific hypersensitivity has been well documented (Seitz et al. 2013).

Most recent work considers drug specific $CD8^+$ T cells as the main, or at least, the first effector cell in maculopapular exanthema. These $CD8^+$ T cells infiltrate positive patch tests to drugs in the few hours after drug application, even before any macroscopic reaction is visible. They reach the skin before the recruitment of $CD4^+$ T cells, that come later and may act more as regulatory T cells (Rozières et al. 2010),(Vocanson et al. 2009).

B.7.2. Drug reaction with eosinophilia and systemic symptoms

The effector reaction in skin lesions of DRESS has some analogy to maculopapular exanthema, but it involves much more complex mechanisms that are not completely understood, namely in which concerns the relative contribution of the culprit drug or of the viral reactivation in the induction of the skin and multisystemic lesions. Very probably, there is a significant interplay between viral reactivation and the T cell response against the culprit drug and/or viral infected cells. DRESS occurs, then, in individuals that are more susceptible to drug-induced immunosuppression or to drug-induced viral reactivation (Descamps and Ranger-Rogez 2014).

B.7.2.1. Exanthema and multisystemic involvement

In DRESS, comparing to patients exposed to the same drug with no adverse effect, a high percentage of activated CLA^+, $CCR10^+$, $CCR4^+$ skin homing T cells are found in the blood. They are both $CD4^+$ and $CD8^+$ T cells, with more frequent $CD8^+$ T cells during the initial phases (Picard et al. 2010),(Hanafusa et al. 2012).

Activated T cells infiltrate the dermis and epidermis, in some cases with such an exuberant density and activation markers that they can mimic a cutaneous T-cell lymphoma (Shiohara et al. 2007),(Walsh and Creamer 2011). These activated T cells have also been found in biopsies collected from other affected organs, namely the liver, kidney or lung (Picard et al. 2010).

The circulating and skin infiltrating T cells, either specific for the drug or for the virus, produce high amounts of pro-inflammatory cytokines and effector molecules. Moreover, drug specific T cell clones isolated from the blood are also rich in perforin and secrete high amounts of IFN-γ (Naisbitt 2004), a cytokine that seems to control the duration and severity of the inflammatory

response (Picard et al. 2010). In agreement, the cytokines INF-γ, TNF-α, IL-6, IL-5 and IL-17E and granulysin are increased in the blood during the acute phase and their increase seems proportional to the severity of skin disease (Hirahara et al. 2010),(Picard et al. 2010),(Saito et al. 2012),(Ogawa et al. 2013). For TNF-α and CCL17, a correlation also seems to exist between their serum levels and HHV-6 reactivation in DRESS patients (Uno et al. 2014),(Ogawa et al. 2014).

In DRESS, T cells secrete IL-5, CCL11/eotaxin and, eventually, also IL-17E, that, together, are responsible for eosinophil recruitment. Eosinophils, present in high amounts in the blood, are recruited and activated both in the skin and systemic organs, where they are considered to mediate tissue damage (Choquet-Kastylevsky et al. 1998),(Poszeczynska-Guigné et al. 2005). Eosinophilia and increased IL-5 reflect a Th2 profile of the T cell reaction, which may be secondary to the high production of CCL17/TARC by dermal $CD1a^+$ cells and dermal dendritic $CD11c^+$ cells, in response either to the virus or to the drug (Ogawa et al. 2013),(Ogawa et al. 2014).

Both IL-5 and CCL17 recruit $CCR4^+$ cells into the skin, and circulating levels of CCL17 were shown to correlate both with the intensity of erythroderma and eosinophil levels in DRESS (Ogawa et al. 2013). However, CCL17 levels do not correlate with the degree of hepatic cytolysis (Ogawa et al. 2013), which is in agreement with the study of Walsh *et al.* (Walsh et al. 2013) showing absence of correlation between the intensity of the exanthema or the presence of exfoliative erythroderma and liver cytolysis. In their group of DRESS patients, liver cytolysis correlated better with an erythema-multiforme–like pattern of exanthema with higher vacuolar degeneration and keratinocyte apoptosis, which are considered to be induced preferentially by $CD8^+$ cytotoxic T cells, as in other forms of drug induced cytotoxicity (Walsh et al. 2013).

Granulysin, a T cell cytotoxic granule involved in keratinocyte apoptosis and epidermal necrolysis in SJS/TEN, is also highly increased in the blood during the acute phase of DRESS, and this increase is proportional to the severity of DRESS (Saito et al. 2012). Therefore, granulysin might be responsible for skin

and, eventually, also liver aggression. Nevertheless, and according to Ogawa *et al.*, granulysin in DRESS is not the product of drug-specific T cells but it is released by virus specific cytotoxic T cells (Ogawa et al. 2013).

B.7.2.2. Anti-drug and anti-viral T-cell responses

In DRESS, a specific immune reaction directed against the drug is confirmed by positive patch tests, observed particularly with carbamazepine and abacavir (Santiago et al. 2010),(Phillips et al. 2002). This mechanism is also supported by the isolation of $CD4^+$ and $CD8^+$ drug specific T cells from the skin, the blood or other organs, sometimes in high amounts (Naisbitt 2004),(Caubet et al. 2011), particularly in the cases of carbamazepine, lamotrigine (Naisbitt 2004) and abacavir. In this latter drug, only $CD8^+$ drug specific T cells were isolated, but these are also the main infiltrating cell in DRESS from abacavir (Phillips and Mallal 2009a).

As an important argument against the predominant contribution of the anti-drug effector T cell response, DRESS does not resolve only by suspending the culprit drug. It may even worsen and may have a prolonged and relapsing course with no further exposure to the initial culprit drug (Cacoub et al. 2011).

On the other hand many data support the viral theory. Multiple and sequential reactivation of various herpes virus, mostly HHV-6, but also HHV-7, EBV and CMV, are documented during the course of DRESS, usually during the 2^{nd} or 3^{rd} week. Viral DNA particles and viral mRNA, sometimes with a very high number of viral copies, are detected by PCR in the blood or bone marrow and, also, in the skin or internal organs involved (Descamps 2013). In situ hybridization techniques or immunocytochemistry directed to the viral antigens also confirm the presence of the virus in the skin and other affected tissues, along with viral specific T cells (Hashizume and Takigawa

2005),(Shiohara et al. 2007),(Eshki et al. 2009),(Picard et al. 2010),(Camous et al. 2012),(Descamps et al. 2013),(Hashizume et al. 2013). Moreover, according to Picard *et al.*, who sequenced the CDR3 region of the TCR in $CD8^+$ T cells in DRESS, a high proportion of the activated T cells in the blood, skin and affected organs share many sequences with the EBV or CMV specific T cells (Hashizume and Takigawa 2005),(Picard et al. 2010).

Therefore, the T cell response against viral infected cells in the skin may be directly responsible for the exanthema. This may explain important similarities between DRESS and a viral disease at its onset and the similarities observed also between other drug induced and viral induced exanthema, both on clinical aspects and histopathology (Hari et al. 1999),(Bellini et al. 2013).

There is no definitive explanation on how viral reactivation and both a specific anti-viral and drug specific T cell responses concur to the cutaneous and multiorgan injury observed in DRESS. Nevertheless, at present, many data support the interplay between these two elements (virus and drug) in this CADR, and there are several possible explanations for this interplay.

One of the first theories is based on the finding of hypogammaglobulinemia and low B-cell counts at the beginning of the DRESS. It is suggested that the drugs, particularly the anticonvulsants, induce immunosuppression with hypogammaglobulinemia and/or an increase of regulatory T cells that suppress the memory T cells involved in the control of viral proliferation (Shiohara et al. 2007),(Takahashi et al. 2009),(Descamps and Ranger-Rogez 2014). Actually, these authors have detected high levels of circulating skin homing Treg cells ($CD4^+CD25^{+bright}Foxp3^+$ and CLA^+CCR4^+) during the acute phase of DRESS (Shiohara et al. 2007),(Takahashi et al. 2009). Immunosuppression would favour viral reactivation considered, according to this theory, responsible for the systemic symptoms, the initial exanthema and its frequent reactivations during the course of the disease (Kano et al. 2004),(Wong and Shear 2004),(Shiohara et al. 2007).

This theory could also explain the long latency period between drug introduction and the first symptoms of DRESS, but most drugs involved in DRESS are not particularly immunosuppressive and most widely used immunosuppressive drugs are not associated with DRESS.

Still, in accord with this theory, after the initial phase with immunosuppression, and particularly after drug suspension, the recovery of $CD4^+$ and/or $CD8^+$ T cells induces an immune reconstitution inflammatory syndrome (IRIS) with damage of the tissues where the virus is localized, as observed in AIDS after highly active anti-retroviral treatment (HAART).

This initial period of immunosuppression might also explain negative lymphocyte transformation tests (LTT) during the acute phase of DRESS, whereas they become positive 5 to 7 weeks after recovery, when a full immune reconstitution occurs, in parallel with the reduction of the circulating skin homing regulatory T cells (Shiohara et al. 2007)(Takahashi et al. 2009). This immunorecovery might also explain the occurrence of enhanced responses to other drugs, originating frequent relapses during the evolution of DRESS, and the higher frequency of autoimmune phenomena that develop, specially, after DRESS resolution (Shiohara et al. 2007),(Pichler et al. 2011),(Chen et al. 2013).

Another theory proposes that virus specific T cells exhibit cross-reactivity with culprit drugs and, therefore, drug exposure expands both viral and drug specific T cells (Shiohara et al. 2007). This has not been proven, but recent studies have indicated that cross-reactivity between viral peptides and certain allogeneic HLA molecules, observed in organ transplantation during T-cell mediated rejection, might also be involved in CADR, where the drug interacts with HLA molecules (Pavlos et al. 2014).

Still, based on the isolation of both drug specific T cells clones and viral specific T cells from the blood and affected organs, and on the presence of viral copies in the circulation and affected tissues, another theory proposed that the culprit

drug induced T cell proliferation, or another direct effect on lymphocytes, which culminated in the reactivation of herpes virus present in a latent state in these T cells. Actually, recent *in vitro* studies with infected lymphoid/lymphoblastoid cell lines have shown that, the addition of amoxicillin, carbamazepine or sodium valproate to these cells induces the proliferation of HHV-6, EBV and CMV (Mardivirin et al. 2009). Moreover, using EBV-transformed B cells from DRESS patients, Picard *et al.* found an increased number of EBV copies in culture in the presence of the drugs causing DRESS (carbamazepine, allopurinol, sulfamethoxazole and sodium valproate), but not in the presence of gentamycin, a drug that has not been reported to induce DRESS (Picard et al. 2010). According to this theory, if sodium valproate is similar to amoxicillin and carbamazepine on viral reactivation, it should be contraindicated in DRESS by aromatic anticonvulsants. This is not consistent with the long standing clinical experience that has shown that sodium valproate is the "safe" alternative drug to be used in cases of DRESS from carbamazepine and other aromatic anticonvulsants (Santiago et al. 2010). Moreover, there are very few cases of DRESS attributed to sodium valproate (Wang et al. 2012) and sodium valproate is not usually included in the lists of drugs causing DRESS (Descamps and Ranger-Rogez 2014), which may also weaken the hypothesis of drug-induced viral reactivation as the main explanation for DRESS.

On the other hand, the capacity of amoxicillin to activate latent herpes virus in T cells *in vitro* (Mardivirin et al. 2010) can explain many clinical cases, where the manifestations of DRESS are enhanced when amoxicillin is used to treat the first symptoms or cases of DRESS that are reactivated when this antibiotic is used to control a secondary infectious complication. By analogy, this theory could explain why, during acute EBV infection, amoxicillin significantly enhances the skin and systemic symptoms of infectious mononucleosis, even though not all individuals develop definitive delayed hypersensitivity to amoxicillin and react to this antibiotic out of the context of EBV infection (Renn et al. 2002),(Jappe 2007),(Mardivirin et al. 2009).

Recently, Hideo Hashizume and collaborators found HHV-6 in circulating monomyeloid precursors, present in high numbers in the circulation of DRESS patients and, later, also in the affected skin. These cells that carry the virus, are highly prone to skin migration due to the high expression of CLA and the skin chemotactic receptors CCR4 and CCR10. Also, they seem to be particularly attracted by a protein of the DAMP family, the high mobility group box-1 (HMGB-1) that is expressed in high amounts in the cytoplasm of keratinocytes in DRESS. This alarmin, produced by keratinocytes, is a non-histone nuclear protein that binds chromatin and regulates transcription in the nucleus. In stressful situations, as observed in the skin of DRESS, HMGB-1 is translocated to the cytoplasm and secreted in high amounts to the extracellular fluid, where it acts as a very potent chemotactic factor for monocytes and myeloid cells. In the skin, these infected monomyeloid precursors can transfer HHV-6 to skin infiltrating $CD4^+$ T cells where the virus proliferates actively (Hashizume et al. 2013). Accordingly, we may speculate that the exanthema in DRESS may represent, initially, a drug specific T cell response that is later reactivated by HHV-6 infection and a subsequent anti-viral immune response.

At present, we still do not to completely understand whether the strong immune activation observed in DRESS is the cause or the consequence of herpes virus reactivation (Rozières et al. 2009b) and this may question the most adequate therapy. Most groups favour immediate drug suspension and use corticosteroids or other immunosuppressive drugs to reduce the immune reactivation causing severe symptomatology, whereas others prefer avoidance of immunosuppressors (Shiohara et al. 2007) and propose antiviral treatment (ganciclovir) and IVIG in severe cases (Descamps et al. 2010),(Criado et al. 2012), although contradictory results have been reported, particularly for IVIG (Joly et al. 2012). Immunosuppressive drugs used at the beginning may enhance viral reactivation but, on the other hand, they seem to protect from delayed autoimmune phenomena, increasingly described after DRESS resolution (Shiohara et al. 2012).

B.7.3. Acute generalized exanthematous pustulosis

Acute generalized exanthematous pustulosis (AGEP) represents a pustular neutrophilic reaction, but there is wide evidence that neutrophil recruitment is due to the effect of drug specific T cells. Lymphocyte transformation tests are often positive, as well as specific drug patch tests. Moreover, patch tests often show a pustular pattern with the typical spongiform intraepidermal pustule, observed during the acute eruption (Schmid et al. 2002),(Schaerli et al. 2004),(Serra et al. 2011).

Drug-specific T cell clones have been isolated from positive patch tests as well as from the blood during the acute phase. They are $CD8^+$ and, mainly, $CD4^+$ memory effector T cells (Britschgi et al. 2001). The $CD4^+$ T cells produce particularly high levels of IL-8/CXCL8, the neutrophil chemokine, and express the chemokine receptor CCR6 (Schaerli et al. 2004), which is the main skin homing receptor for Th17 cells. The capacity of these T cells to recruit and boost neutrophilic inflammation seems common to AGEP and other neutrophilic auto-inflammatory dermatosis, namely generalized pustular psoriasis and Beçhet's disease (Keller et al. 2005), and may be dependent on deficiencies of IL-36RN (IL-36 receptor antagonist) (Sugiura et al. 2013),(Navarini et al. 2013).

Drug specific T cells also exhibit cytotoxicity against drug laden target cells, mainly through perforin and granzyme B (Schmid et al. 2002), and also through granulysin and FasL (Speeckaert et al. 2010). The involvement of NK cells and granulysin does not seem to be very significant in this CADR (Schlapbach et al. 2011b), but initial cytotoxicity may be important for epidermal aggression before the pustule formation (Schaerli et al. 2004).

Drug specific T cells secrete mainly type 1 cytokines (IFN-γ, TNF-α and GM-CSF), associated in some cases with IL-5, responsible for eosinophilia observed in about one third of AGEP patients (Britschgi et al. 2001),(Speeckaert et al. 2010).

The main particular characteristic of the drug specific T cells in AGEP is their capacity to produce high quantities of IL-8/CXCL8, along with GM-CSF and IFN-γ, which actively contributes to the recruitment of neutrophils to the epidermis (Schaerli et al. 2004). Through the neutrophil receptor CXCR1, the chemokine IL-8/CXCL8 attracts the neutrophils to the skin and, mainly through the CXCR2, this chemokine and GM-CSF prevent neutrophil apoptosis and prolong their survival in the skin, allowing the formation of the spongiform pustule. Interestingly, pustules seem to form mainly at sites of previous keratinocyte damage (Schaerli et al. 2004), very probably due to the cytotoxicity of the drug specific T cells referred above.

More recently, Th17 cells and IL-22 were found to be significantly and particularly elevated in this pattern of CADR (Kabashima et al. 2011). Along with IL-8/CXCL8, IL-17 and IL-22 have a synergic action on keratinocytes which further secret IL-8/CXCL8 and, also, IL-36 (Lowes et al. 2013). As both T cells and keratinocytes secret IL-8/CXCL8 and T cells also express the receptor CXCR1, very probably a pro-inflammatory amplification loop develops, with further T cell and neutrophil infiltration (Britschgi and Pichler 2002),(Rozières et al. 2009b),(Speeckaert et al. 2010).

Interleukin-36, a member of the IL-1 family secreted by injured keratinocytes as a defensive response against exogenous aggressions or microbes, can also participate in this inflammatory amplification loop. IL-36 attracts and activates neutrophils that infiltrate the epidermis. Moreover, this cytokine particularly activates skin DC and induces IL-23 production, which is the main DC-derived cytokine necessary for the differentiation of T cells into Th17 (Lowes et al. 2013).

Actually, IL-36 may further establish some link between AGEP and generalized pustular psoriasis, although this relation still remains controversial (Sugiura et al. 2014),(Navarini et al. 2014). The absence of an IL-36 antagonist or a mutation in IL-36RN gene that controls the pro-inflammatory activity of

IL-36 was found in a minority of AGEP patients, as well as in a few families with generalized pustular psoriasis (Navarini et al. 2013),(Lowes et al. 2013), particularly those without psoriasis vulgaris (Sugiura et al. 2013). Although AGEP may share this mutation with some cases of familiar generalized pustular psoriasis and although there are clinical and histological similarities between the 2 diseases, the acute course of AGEP, its clear relation with drugs other than those classically inducing pustular psoriasis, the absence of personal or family history of psoriasis in the great majority of AGEP patients (Sidoroff 2012) and the reactivity in drug patch tests, usually allow a distinction between AGEP and generalized pustular psoriasis (Serra et al. 2011),(Navarini et al. 2014). Nevertheless, Sugiura and co-workers recently described two monozygotic twins with generalized pustular eruptions, once triggered by amoxicillin confirmed with a positive patch test, but who kept having pustular eruptions with no further amoxicillin exposure and who were, therefore, considered to have a form of generalized pustular psoriasis (Sugiura et al. 2014).

With some difficulty in establishing a sharp limit between AGEP and generalized pustular psoriasis we may, nevertheless, consider that both diseases share a common inflammatory anomalous pathway involving IL-8/CXCL8, IL-36 and IL-17, and they may, eventually, coexist in the same patient (Serra et al. 2011),(Sugiura et al. 2014),(Navarini et al. 2014).

AGEP is, therefore, a very peculiar neutrophilic CADR driven by drug specific T cells that selectively attract and activate neutrophils in the skin, a type IVd hypersensitivity reaction (Lerch and Pichler 2004). It often develops within a very short period after drug administration, sometimes less than 24h. Moreover, when considering responsible drugs, some that seldom induce other types of exanthematous CADR are typically involved in AGEP, like terbinafine and diltiazem (Sidoroff 2012). No particular effect of these drugs on neutrophil activation has been described, except that terbinafine, *in vitro*, enhanced the production of IL-8/CXCL8 in a human monocytic cell line, the THP-1 (Mizuno et al. 2010).

Some drugs, like antibiotics, induce both AGEP and non-pustular type IV reactions. On the other hand, drugs mostly involved in SJS/TEN or DRESS (allopurinol and anticonvulsants) seldom cause AGEP (Sidoroff 2012). Therefore, this reaction pattern does not seem to dependent entirely on the drug. It is very probably dependent on concomitant factors, still unidentified, or on an individual background (genetic or not), that promote the neutrophilic inflammation. This immune reaction, representing a very interesting cross-talk between keratinocytes and T cells mediated by CXCL8, IL-17, IL-22 and/or IL-36, can bring important insights on the knowledge of the pathophysiology both of AGEP, pustular psoriasis and other neutrophilic diseases. It can also open new areas of investigation to find more targeted therapies for these diseases.

B.7.4. Stevens-Johnson syndrome/Toxic epidermal necrolysis

In exanthematic necrolysis, as Roujeau calls the group of diseases characterized by more than 1% epidermal necrolysis and skin detachment, including SJS, SJS/TEN overlap and TEN, there is extensive epidermal necrolysis (apoptosis and necrosis) induced by T cells and their mediators (Roujeau et al. 2011).

T cell participation in SJS/TEN was initially questioned, as many skin biopsies contain very few lymphocytes in the dermis. This is explained as dermal infiltrating lymphocytes quickly migrate to the epidermis, where they exert their cytotoxic effect, and they are mainly found, in high numbers, in the blister fluid of these patients (Correia et al. 1993). Today there are strong arguments confirming the involvement of drug specific T cells also in SJS/TEN, particularly $CD8^+$ T cells, which have been shown to recognize drugs bound to HLA-class I molecules in target cells (Nassif et al. 2004a).

B.7.4.1. Main effector cells

In SJS/TEN, $CD8^+$ T cells that infiltrate the dermis, epidermis and are found in the blister fluid are generally considered the main effector T cells in this severe CADR. These $CD8^+$ T cells are mostly $CD8^+CD56^+$ cytotoxic T lymphocytes (CTL). They specifically recognize the drug and kill autologous keratinocytes, or other cells, expressing HLA molecules combined with the culprit drug. Consequently, some keratinocyte aggression occurs in a drug and HLA restricted manner (Nassif et al. 2004a), as shown for carbamazepine-induced TEN in HLA-B*15:02 patients (Pavlos et al. 2014), but keratinocyte apoptosis further progresses in the absence of the drug (Chung et al. 2008).

In SJS/TEN, the dermal ambience rich in dendritic cells and monocytes that express high levels of activation, namely CD80/CD86 and CD137L, seem to expand particularly $CD8^+$ T cells expressing complementary receptors, respectively, CD28 and CD137. Therefore, upon engagement of the TCR-HLA and the costimulatory receptor (CD80/86-CD28), $CD8^+CD137^+$ T cells are activated through CD137/CD137L, become protected from death, proliferate and increase their cytotoxic potential (Tohyama and Hashimoto 2012).

Natural Killer (NK) cells represent a significant percentage in the cellular infiltrate in TEN (±13% are $NKp46^+$ cells), and they may also play an important part in keratinocyte necrolysis (Schlapbach et al. 2011b).

Other subtypes of T cells can participate in this effector phase. $CD4^+$ Th17 cells, expressing the skin homing receptors CLA^+CCR4^+, are increased in the circulation and also in the blister fluid, and its value in the blister fluid augments proportionally to the body surface area involved (Teraki et al. 2013). Nevertheless, it is not completely understood how they collaborate in epidermal cytolysis.

B.7.4.2. Mechanisms of keratinocyte apoptosis

Drug specific $CD8^+$ cells (and eventually NK cells) exert a direct cytotoxic effect, by cell-to-cell contact or close proximity. Perforin, granzyme B and granulysin kill keratinocytes whose HLA-class I was modified by the drug, allowing NK/$CD8^+$ T cell contact. But, the relatively small number of cytotoxic cells found in SJS/TEN lesions could hardly explain the exuberant epidermal necrolysis (Roujeau et al. 2011),(Araujo et al. 2011). Therefore, many cytotoxic molecules have been the object of study as a possible amplifier of the keratinocyte death in TEN, namely Fas/FasL, nitric oxide, TNF-α and related death molecules and, recently, also granulysin.

The suicidal interaction between Fas (CD95) and FasL (CD95L), in soluble or membrane forms, was implicated in keratinocyte necrolysis. Soluble FasL increases in the blister fluid and, in the very early stages of TEN, FasL levels are also increased in the blood (Abe 2008),(Saito et al. 2013). Fas is upregulated in keratinocytes but cells expressing FasL do not co-localize with keratinocytes expressing Fas in skin biopsies of TEN which, therefore, does not support this is a major mechanism of cell death in TEN (Nassif et al. 2004b). Moreover, intravenous immunoglobulins (IVIG) used in the treatment of TEN, based on their capacity to neutralize FasL, had irregular results (Faye and Roujeau 2005), (Lee et al. 2013a). Also, the use of antibodies anti-Fas in animal models did not prevent keratinocyte apoptosis (Allanore and Roujeau 2007).

TNF-α and other death receptor ligands that use pathways similar to Fas/FasL are also found in high amounts in blister fluid in TEN, particularly TRAIL produced by $CD8^+$ T cells and TWEAK produced by $CD14^+$ macrophages/monocytes and $CD1a^+$ DC. Although they may also contribute to keratinocyte death, these mediators are not considered the main effectors in TEN (Araujo et al. 2011).

At present, the main candidate for disseminated epidermal necrolysis is granulysin. It induces keratinocyte apoptosis both in *in vitro* models and, also,

in vivo, after intracutaneous injection in mice. Moreover, granulysin depletion was shown to abrogate the capacity of blister fluid to induce keratinocyte apoptosis, both *in vitro* and after intradermal injection in mice (Chung et al. 2008).

Granulysin is detected in blister fluids in very high amounts, more than 300-fold higher than in bullae from burned patients. These concentrations were shown to be higher than the ones needed to induce keratinocyte apoptosis *in vitro* or *in vivo* (Chung et al. 2008). Moreover, granulysin concentrations in blister fluid show a positive correlation with the severity of skin detachment, serum granulysin concentrations increase from the very early phases of SJS/TEN and its levels are positively correlated with the severity of the reaction (Abe et al. 2009),(Saito et al. 2013). In agreement with this data, granulysin producing cells ($CD8^+$ T cells and NK cells) accumulate in areas of more extensive epidermal aggression, as observed on histopathology (Schlapbach et al. 2011b), although it is known that granulysin cytotoxic activity can affect keratinocytes at a considerable distance. At present, there is no available drug or monoclonal antibody that can block the effect of this important mediator, but it might be a good promise for the very early, targeted treatment of TEN.

The process of keratinocyte cell death can also involve IFN-γ and TNF-α produced by cytotoxic T cells (Fig. B.17). These cytokines enhance disease spreading by turning keratinocytes more susceptible to apoptosis and equipping them with the capacity to induce apoptosis of "innocent bystander" neighbouring keratinocytes (Nassif et al. 2004a). Even without carrying the culprit drug, INF-γ stimulated keratinocytes upregulate HLA-I molecules and death receptors on their cellular membrane and produce high amounts of nitric oxide. Consequently, keratinocytes become more susceptible to killing, both by $CD8^+$ T cells and their mediators and, also, by TNF-α and related death ligands.

Moreover, damaged keratinocytes also produce TNF-α, becoming the target of their own secreted cytokines (Nassif et al. 2004a),(Araujo et al. 2011),(Viard-Leveugle et al. 2013). The importance of TNF-α in this vicious cycle of

keratinocyte death amplification is supported also by some successful, although isolated reports on the use of anti-TNF drugs in TEN (Hunger et al. 2005),(Wojtkiewicz et al. 2008),(Scott-Lang et al. 2012),(Viard-Leveugle et al. 2013). Particularly for infliximab, it has been reported that skin blistering can cease within 24 hours of administration (Scott-Lang et al. 2012). On the opposite, thalidomide, also known to inhibit TNF-α, showed TEN aggravation (Schwartz et al. 2013a) and an European multicentre clinical trial had to be suspended prematurely.

B.7.4.3. Pro-inflammatory amplification loop

Once initiated, keratinocyte apoptosis and skin inflammation progresses in SJS/TEN with a variable rapidity, and may induce epidermal necrosis in a relatively reduced body surface area or quickly extend to cover almost the full body surface. Reasons for the different velocity and extension of progression are not completely explained, although amplification inflammatory loops and absence of T regulation have been clearly identified in SJS/TEN.

Keratinocytes stimulated by T cells, namely by IFN-γ, secrete CCL27/CTACK, which further attracts $CCR10^+$ cutaneous memory T cells (Tapia et al. 2004). Damaged keratinocytes also liberate many cytokines involved in the inflammatory amplification loop, namely IL-18 (Nassif et al. 2004b). Moreover, damaged keratinocytes overexpress and liberate endogenous molecules, "alarmins", like S100A protein, α-defensins, and HMGB-1, which are increased either in the blister fluid and/or in the serum of patients during the acute phase of TEN (Nakajima et al. 2011). These alarmins, recognized as DAMP, recruit inflammatory cells and activate innate immunity (Tohyama and Hashimoto 2012). HMGB-1, found in skin biopsies mainly near keratinocytes showing vacuolization or apoptosis, may also be further involved in the regeneration of the epidermal layer (Nakajima et al. 2011).

In SJS/TEN, epidermal necrolysis progression may also be due to the absence of an effective T cell regulatory response. Although at the beginning of TEN, the number of circulating regulatory T cells does not seem to be decreased, comparing to healthy individuals, their T regulatory function is profoundly impaired. They are unable to inhibit proliferation and cytokine production when specific T cells are exposed to the drug (Takahashi et al. 2009). Also, these Treg cells do not migrate efficiently to the skin. Actually, $CD4^+CD25^+Foxp3$ regulatory T cells are almost absent in skin lesions of TEN, particularly in regions of high $CD8^+$ T cell infiltration and marked apoptosis (Takahashi et al. 2009).

Moreover, $CD16^+$ monocytes/macrophages that accumulate in the skin in TEN are highly involved in the inflammation and seem unable to produce IL-10 (Tohyama and Hashimoto 2012), a cytokine that might activate regulatory T cells and downregulate the on-going inflammation and necrolysis in SJS/TEN. Actually, in animal models of drug induced TEN, epidermal necrolysis is significantly reduced in the presence of $CD4^+$ Treg cells, whereas depletion of these $CD4^+$ T cells enhances necrolysis (Azukizawa 2011).

As a summary, SJS/TEN represents a complex interplay between drug specific $CD8^+$ T cells, other cytotoxic cells and their cytotoxic mediators and the resident keratinocytes, which are the target of cytotoxicity. Stimulated and damaged keratinocytes also enhance the reaction, both by turning neighbouring cells more susceptible to apoptosis and by secreting pro-inflammatory cytokines that call other inflammatory and cytotoxic cells to the "battle field". This seems to happen in an ambience where immune regulation is significantly deficient, namely due an inefficient activity of $CD4^+Foxp3^+$ T cells.

B.7.5. Fixed drug eruption

Fixed drug eruption is a very peculiar localized drug hypersensitivity reaction. It is restricted to very specific and well-demarcated areas of the skin or mucosa but, thus far, there is no explanation for the involvement of certain areas whereas the neighbouring skin is completely spared. Previous skin lesions become inflamed shortly on drug exposure, due to the reactivation of drug specific T cells that persist in residual lesions for many years (Shiohara 2009),(Rozières et al. 2009b). On a future drug exposure, some residual lesions can enlarge and new lesions can occur, but this CADR generally keeps as a localized reaction.

$CD8^+$ T cells, with a phenotype resembling memory effector $CD8^+$ T cells that are present at mucosae near sites of pathogen entry, persist in the residual lesional epidermis in abnormal numbers, alongside the dermal epidermal junction. They were observed for more than 4 years after clinical resolution of the acute lesion, even in the absence of further drug exposure. According to the hypothesis of Shiohara and co-workers, these cells may be committed to anti-HSV defence, explaining the more frequent occurrence of fixed drug eruptions at the places of previous herpetic lesions (Shiohara and Mizukawa 2012).

These $CD8^+$ T cells that express the skin homing receptor (CLA^+) and bind to keratinocyte E-cadherin by their integrin $\alpha 3\beta 7$ (CD103), are kept in the epidermis where their survival is constantly enhanced by IL-15, constitutively produce by keratinocytes (Shiohara et al. 2002),(Mizukawa et al. 2008),(Shiohara 2009).

These resting $CD8^+$ T cells share surface and activation markers with NK cells, namely the CD69 molecule, and show some specific anti-drug cytotoxicity. In the resting plaque, these intraepidermal T cells stay along the basal layer and do not harm the neighbouring keratinocytes that are protected from apoptosis. But, within a few hours upon exposure to the culprit drug, these resting or "pre-activated" T cells migrate to the upper epidermal layers, become activated and initiate a process of epidermal aggression, very similar to TEN. Cytotoxic T

cells quickly upregulate mRNA for IFN-γ, secrete high amounts of this this cytokine, which turns keratinocytes more susceptible to apoptosis by the expression of Fas, and CTL initiate apoptosis and necrosis of neighbouring keratinocytes by their cytotoxic mediators, including FasL, TNF-α, perforin, granzyme B and granulysin (Choi et al. 2006),(Mizukawa et al. 2008),(Shiohara 2009),(Shiohara and Mizukawa 2012).

Apart from intraepidermal T cells, other $CD8^+$ effector T cells are recruited from the circulation and participate in the epidermal aggression (Mizukawa et al. 2008),(Shiohara 2009). $CD8^+$ T cells and NK cells expressing high levels of granulysin are initially observed at the dermal-epidermal interface, where vacuolar degeneration is most significant, and further invade the epidermis and accumulate particularly near vesicles or blisters (Schlapbach et al. 2011b). Other cells, namely neutrophils and eosinophils, may also infiltrate the dermis of fixed drug eruptions, very occasionally being the predominant cell in this type of CADR (Shiohara and Mizukawa 2012).

Contrary to TEN, where regulatory T cells are almost absent from the dermis (Shiohara 2009), very soon as the fixed drug eruption progresses, $CD4^+$ T cells, particularly $CD4^+CD25^{+hi}$ regulatory T cells, are recruited to the skin and localize preferentially in the dermis. These $CD4^+$ T cells downregulate the reaction, both by secreting IL-10 and TGF-β and, as they migrate to the epidermis, also by direct cell-cell contact with the effector T cells. In this way the progression of apoptosis is quickly stopped and becomes limited (Teraki et al. 2006).

These regulatory $CD4^+$ T cells also seem to be involved in the process of desensitization in fixed drug eruption, upon repeated frequent exposures to low doses of the drug (Teraki and Shiohara 2004).

The presence of the “pre-activated” T cells in the residual lesional epidermis can explain why patch testing is negative in normal skin, whereas, a few hours after application of the culprit drug on previously involved skin, the residual

lesion suffers reactivation with the clinical aspect and histopathology typical of a fixed drug eruption (Shiohara 2009),(Andrade et al. 2011).

Although some authors suggest these lesions can be also reactivated by non-specific stress or danger signals (Shiohara et al. 2002), in our experience, lesional reactivation by patch testing is drug specific, often allowing the confirmation of the culprit drug. Moreover, lesional patch testing can also be used to study cross reactions with relevant results for advising patients on future use of drugs (Gonçalo et al. 2002),(Cravo et al. 2007),(Andrade et al. 2011).

B.8. Delayed cutaneous adverse drug reactions: translation of pathophysiologic mechanisms to clinical practice

Although much has still to be investigated, it is well documented the participation of drug reactive T cells in all the different delayed CADR referred above. In each clinical phenotype there is a predominant involvement of a subphenotype of T cells (CD4+, CD8+, CD56+, Th1, Th2, Th12, Tc1, Treg) that significantly influences the cutaneous expression, depending on its main effector function. Secreted cytokines and chemokines and effector cellular equipment, acting by cell-cell contact or at a distance (perforin, granzyme B, granulysin, TNF-α, INF-γ, IL-2, IL-5, IL-13, IL-17, IL-22, and others), dictate a more cytotoxic response on skin resident cells, as in SJS/TEN or fixed drug eruption, or a major dermal inflammation with frequent extension to the epidermis, as in maculopapular exanthema or DRESS. Different chemokines, secreted in the skin setting, selectively attract different phenotypes of inflammatory cells (IL-8/CXCL8 and neutrophils, IL-5 and eosinophils, CCL17, CCL22 and CCL27 and memory T cells), which may significantly modify the clinical expression of the CADR, as in AGEP. “Alarmins” (HMGB-1, granulysin) further activate the innate and adaptive immunity, which, together, may amplify or, eventually, dampen the cutaneous reaction. The

different combinations of these effector pathways determine the heterogeneity of clinical patterns of delayed CADR, observed in the clinic.

A detailed knowledge of these pathophysiologic mechanisms has brought new insights into the handling of patients with delayed CADR in our daily practice. Some are already regularly being implemented, others are still in the field of research, but others may soon translate into clinical practice. For the moment, preventive measures based on known risk factors and improvement on diagnostic tests, represent the main advance. New therapeutic attitudes have had a limited benefit, particularly in the treatment of the more severe CADR.

B.8.1. Recommended preventive measures based on the Knowledge of pathomechanisms

For the moment, most preventive attitudes are dependent on the known predisposition of certain HLA genotypes to severe CADR induced by specific drugs.

At present, HLA-B*57:01 testing is regularly performed worldwide before abacavir therapy in HIV infected patients, often using newer and quicker techniques (Dello Russo et al. 2014), and this measure has almost completely abrogated the severe hypersensitivity syndrome induced by this drug. This same haplotype also seems to predispose to drug induced liver injury (DILI) by flucloxacillin (Kaniwa and Saito 2013), although not significantly in HIV-infected patients (Vera et al. 2013),(Phillips and Mallal 2013). Interestingly this HLA genotype in HIV patients is one of those significantly associated with less disease progression to AIDS and the state of Elite controllers, suppressors or chronic progressors, as $CD8^+$ T cells from these HLA-B^*57^+ patients have an effective response against immunodominant epitopes of both wild type and mutants of HIV (Pohlmeyer et al. 2013). This data is consistent with the possible cross-reactions between anti-viral T cell responses and the immune reaction against certain drugs combined with HLA.

Testing for HLA genotypes before treating with other drugs has not such a strong and worldwide recommendation, either because the association carries a lower risk, as HLA-B*58:01 and allopurinol induced CADR, or because the risk is limited to some populations, as HLA-B*15:02 and carbamazepine induced SJS/TEN in Han Chinese. Nevertheless, its use in these populations at risk is being implemented with a significant reduction in severe CADR. In the near future, extension of genotyping for HLA-A*31:01 in Europeans and Japanese before carbamazepine treatment, or for HLA-B*13:01 before using dapsone in Chinese leprosy patients, and still for newly discovered risk associations in CADR, and other ADR, namely in DILI, agranulocytosis, drug-induced lupus (Pavlos et al. 2014), may certainly translate into significant reduction of severe ADR from the more frequent drugs.

Another interesting preventive recommendation is to avoid amoxicillin in DRESS and, eventually, in patients with infections from herpes virus, based on the its capacity to enhance the proliferation of herpes virus in lymphoid/lymphomononuclear cells in DRESS and its association with relapses or possible induction of DRESS (Mardivirin et al. 2009),(Mardivirin et al. 2010).

B.8.2. Improvement of diagnostic tests for determining the culprit drug using data from pathomechanisms

When facing the patient with a delayed CADR, determination of the culprit drug relies mostly on chronological and clinical data. As cutaneous tests only can be performed weeks after complete resolution of the CADR, if there is an urgent need to confirm the culprit drug, *in vitro* tests may be used during the acute phase, and also retrospectively. Tests based on T cell activation, namely *in vitro* lymphocyte stimulation or proliferation tests (LST) or the ELISpot, have significantly benefited from the knowledge of more details in the pathomechanisms of CADR.

The classical T cell proliferation tests, which evaluated incorporation of ^{3}H-thymidine in cultured lymphocytes and took several days to get the results, are being replaced by tests that evaluate many other parameters of T cell activation by drugs. Using flow cytometer it is easy and quicker to observe the effect of in vitro exposure of circulating lymphocytes to the drug, namely by the expression of new phenotypic markers of activation or by intracellular staining for cytokines (INF-γ, IL-2, IL-5, IL-13) and effector molecules (granulysin or granzyme B), that are known to be involved in the pathomechanisms of CADR. Although already performed in many laboratories, these tests still need standardization in procedures and to establish the ideal dose for testing each drug and the cut-off of stimulation to consider a positive result.

The ELISpot test measures the number of "coloured" spots of different diameter in the petri dish, corresponding to the number of T cells that, upon exposure to the drug *in vitro*, secrete relevant quantities cytokines or effector molecules, which are revealed by ELISA. In its initial steps, only INF-γ was tested, but ELISpot has been successfully enriched with the measurement of other effector chemicals in addition to INF-γ, namely IL-5 and IL-13, (Ebo et al. 2011),(Martin et al. 2010) and, recently, also granzyme B (Bensaid et al. 2012). New candidates molecules to evaluate in the ELISpot might be granulysin and, also, IL-17 and IL-22, the latter particularly in AGEP.

Each of these *in vitro* tests, in its own, has a low sensitivity but their combination can achieve high sensitivity and specificity. In a recent study in SJS/TEN, using LST with intracellular staining for granulysin in circulating $CD4^{+}$ T cells combined with ELISpot detecting INF-γ and granzyme B, 80% sensitivity with 95% specificity was achieved, which is very good for an *in vitro* test (Porebski et al. 2013).

B.8.3. Therapy of delayed cutaneous adverse drug reactions: modest benefit from the knowledge of pathomechanisms

The therapy of CADR relies mostly on early suspension of the culprit drug(s) and symptomatic treatment of CADR manifestations, often using non-specific anti-inflammatory drugs, like the topical or systemic corticosteroids (Bircher 2012).

The use of systemic corticosteroids is nevertheless questioned in severe CADR as DRESS or SJS/TEN, but the alternative drugs suggested on the basis of pathomechanisms have not shown very promising results, for the moment.

In DRESS, corticosteroids reduce the symptoms but they are often associated with relapses when trying to tapper the dose. During the immunosuppressive phase of DRESS, corticosteroids may further potentiate this state, which is, consequently, associated with an increased viral load for HHV-6 and CMV. Viral proliferation and dissemination to the skin and other organs may affect the extension and severity of the tissue infection and, when the corticosteroid dose is reduced, the antiviral T cell response will become more prominent and reactivate cutaneous lesions and multisystemic disease (Shiohara et al. 2012). Therefore, if used in DRESS, corticosteroid tapering has to be very slow, eventually combined with antiviral drugs, like ganciclovir/valganciclovir or cidofovir (Criado et al. 2012),(Shiohara et al. 2012),(Descamps and Ranger-Rogez 2014). Nevertheless, preliminary results seem to show that corticosteroids used in the early phases of DRESS may prevent late autoimmune complications.

In SJS/TEN, early drug suspension and supportive care therapy, taking into consideration the management of fluid and electrolytic balance, early detection of infection, prevention of skin and mucosae complications and good wound healing facilities, are the mainstay of treatment (Harr and French 2012). Corticosteroids in pulse therapy, regularly used in our department, was shown to be effective also in a study in Gröningen using 1.5mg/kg dexamethasone in 3 consecutive days (Kardaun and Jonkman 2007), but their prolonged use is

highly contraindicated. The IVIG or cylosporin A, as single or add-on therapies, have shown favourable results with reduction in expected mortality, but these results have not always been reproduced (Harr and French 2012). Based on the pathogenic importance of TNF-α as a mediator of keratinocyte necrolysis, TNF-α antagonists, particularly infliximab, were shown to be effective in TEN, occasionally stopping disease progression in 24 hours (Hunger et al. 2005),(Wojtkiewicz et al. 2008),(Scott-Lang et al. 2012), but larger studies are still needed. So, for the moment, there is no standardized therapy for SJS/TEN and mortality is still high, with the SCORTEN has a relatively good predictive score.

Although, thus far, therapy has been the area of the study of CADR that has shown the least progression, we may expect that an increasing knowledge of pathomechanisms involved in CADR, combined with the development of new drugs that interfere more precisely with immunologic reactions, and the discovery of relevant targeted therapies, may facilitate the handling of severe CADR in the near future.

C.
OBJECTIVE AND DESIGN OF THE STUDY

C. OBJECTIVE AND DESIGN OF THE STUDY

The objective of the present work is to contribute with some data from our basic research and clinical investigation, for a better understanding of some particular aspects of the immune pathophysiologic mechanisms involved in delayed CADR.

Based on the similarities between ACD and delayed CADR, previously mentioned, we applied the rational for thinking and investigating pathophysiologic mechanisms and diagnostic procedures in ACD to the study of delayed CADR, expecting to obtain results that may eventually contribute to take better decisions in which concerns diagnostic, therapeutic, prognostic and/or preventive measures in delayed CADR.

This work is made possible thanks to the regular study of patients with delayed CADR in the Department of Dermatology of the University Hospital of Coimbra. Most patients are observed in the emergency unit and, then, studied and treated as in-patients or followed closely as outpatients. This has given the dermatologists of the Department an extensive experience in this field and a good training for the registration of precise clinical aspects of CADR and its temporal relation with administered and potential culprit drugs. This allows a good characterization of the phenotypic patterns of the CADR and the determination of the culprit drug, based mainly on clinical and temporal criteria according to the French Pharmacovigilance system (Bégaud et al. 1985)(Moore et al. 1985). Collected data on drug imputation is also compatible with the Naranjo and WHO criteria for diagnosing adverse drug reactions (Naranjo et al. 1992),(Edwards and Biriell 1994) .

During the last years, several reports on CADRs have been published by our group, dealing mainly with the clinical presentations and main responsible drugs involved in severe adverse drug reactions, namely DRESS, SJS/TEN (Santiago et al. 2008),(Brinca et al. 2011), and other patterns of CADR, like fixed drug eruption and reactions from EGF-R inhibitors (Andrade et al. 2011),(Santiago et al. 2011).

Much of the work has also been conducted on the use of patch testing in delayed CADR, performed as a diagnostic test by analogy with ACD. We collaborated in establishing European guidelines for performing skin tests in CADR (Barbaud et al. 2001a) and in evaluating their usefulness in different patterns of drug reactions, with particular emphasis on DRESS (Santiago et al. 2010), maculopapular exanthema (Gonçalo et al. 1999)(Pereira et al. 2011), fixed drug eruption (Gonçalo et al. 2002),(Cravo et al. 2007),(Andrade et al. 2011), AGEP (Serra et al. 2012),(Serra et al. 2011), TEN (Gonçalo et al. 2010), and drug photosensitivity (Gonçalo et al. 1992)(Oliveira et al. 1996),(Canelas et al. 2010),(Cardoso et al. 2009).

The present work, as a continuation of previous clinical studies, consisted on several separate but inter-related investigations:

1. A histopathologic evaluation of the skin lesions in DRESS was performed to characterize better this severe pattern of CADR, frequently observed in our clinic, and where we had previously shown the involvement of drug-specific delayed hypersensitivity mechanisms by patch testing. It was our objective to find specific histologic aspects of skin lesions, which might allow confirm the diagnosis of DRESS, and understand better the type of skin aggression induced by effector cells. As a secondary objective, we intended to correlate histologic parameters with severity aspects in DRESS, with the specific drugs or

pharmacologic groups of drugs that cause this syndrome, or with a concomitant viral reactivation (D);

2. An histopathologic study of positive patch tests with drugs in different drug eruptions was performed in order to understand how they reproduce the CADR and, therefore, to confirm the utility of patch testing as a specific diagnostic test and, also, as a possible tool to understand pathophysiologic mechanisms involved in the effector phase of each pattern of CADR (E);

3. Genetic studies, particularly the characterization of the HLA genotype was performed to evaluate risk factors associated with severe CADR from allopurinol, one of the drugs most frequently involved in severe drug reactions in our population (F).

In order to fill some gaps in our knowledge of pathophysiologic mechanisms, namely in which concerns the initiation of the CADR, we performed basic research to evaluate the *in vitro* effect of drugs causing delayed immune mediated CADR on dendritic-like cells, cells that usually suffer activation when exposed to sensitizing chemicals and that are relevant in the process of antigen presentation in ACD (G). This investigation was a continuation of previous *in vitro* research studies on the mechanisms of antigen presentation in ACD, which had shown a direct stimulating effect of contact sensitizers on dendritic cells. These studies were performed at the Centre for Neurosciences, University of Coimbra and the Institute for Biomedical Investigation of Light and Image (IBILI), FMUC and have been the subject of several publications in the last years (Cruz et al. 2002),(Cruz et al. 2004),(Vital et al. 2005),(Cruz et al. 2005),(Matos et al. 2005a),(Matos et al. 2005b),(Cruz et al. 2007),(Neves et al. 2008), (Francisco et al. 2010),(Neves et al. 2011),(Martins et al. 2012),(Neves et al. 2013).

In the present *in vitro* study, we evaluated the effect of systemic drugs that cause delayed CADR on THP-1 dendritic-like cells, with the objective of understanding if, like contact sensitizers, these systemic drugs also activate DC and, therefore, initiate an innate immune response that may, consequently, enhance antigen presentation and the following adaptive immune reaction responsible for the CADR.

D.
CLINICAL STUDIES
I - HISTOPATHOLOGY OF DRUG REACTION WITH EOSINOPHILIA AND SYSTEMIC SYMPTOMS (DRESS)

D. CLINICAL STUDIES. I - HISTOPATHOLOGY OF DRUG REACTION WITH EOSINOPHILIA AND SYSTEMIC SYMPTOMS (DRESS)

D.1. Introduction

Drug reaction with eosinophilia and systemic symptoms (DRESS) was initially described in association with anticonvulsant hypersensitivity, as an exfoliative erythroderma with a dense mononuclear skin infiltrate, sometimes with atypical cells, which motivated the designation of drug-induced pseudolymphoma (Husain et al. 2013). At present, many drugs other than anticonvulsants are associated with this syndrome and the pseudolymphomatous histological pattern is not so frequently reported. Therefore, the preferred designations for this CADR are DRESS, mainly used in Europe, and DIHS (drug induced hypersensitivity syndrome), used mainly in Asia.

DRESS presents almost always with an exanthema, an exfoliative dermatitis or a generalized maculopapular symmetric eruption (more papular/urticarial or more macular/morbiliform), similar to a drug induced maculopapular exanthema or a viral exanthema (Lee et al. 2012a). There are very few detailed reports on the histopathology of the skin involvement and no specific histologic hallmark has been described in DRESS. Most reviews on this CADR refer briefly a dermal-epidermal infiltrate with lymphocyte exocytosis, spongiosis, basal keratinocyte vacuolization and scattered apoptosis of keratinocytes (Chiou et al. 2008),(Chen et al. 2010),(Walsh et al. 2013),(Husain et al. 2013),(Walsh and Creamer 2011),(Hanafusa et al. 2012). These findings are not significantly different from those usually described in maculopapular exanthema with no, or few, systemic symptoms (Naim et al. 2011),(Seitz et al. 2013). Like in histology, there are no special clinical diagnostic features that

allow the definitive distinction between the skin eruption of a CADR presenting as a simple maculopapular exanthema and a maculopapular generalized eruption within the context of DRESS. Actually, there seems to be a continuum between maculopapular exanthema, with no systemic symptoms or only discrete extracutaneous involvement, and DRESS with a skin and multi-systemic reaction.

In a recent report, data on the histology of DRESS shows a possible correlation between the intensity of the skin lesions observed on histology and the systemic manifestations of DRESS, namely between keratinocyte necrosis and liver cytolysis (Walsh et al. 2013).

The objective of the present work is to perform a more detailed description of the histopathologic changes observed in the exanthema of DRESS, find histologic aspects that may reinforce the diagnosis of DRESS or histopathologic markers that correlate with particular aspects of this CADR (culprit drug, viral infection, severity of systemic manifestations). Histopathology may also contribute to the understanding of how effector cells induce skin aggression.

D.2. Methods

D.2.1. Collection of clinical and laboratory data

Among 223 patients with severe or potentially severe drug eruptions hospitalized at the Department of Dermatology of the University Hospital of Coimbra in the last 5 years (2008-2013), 33 patients (14.8%) fulfilled the RegiSCAR criteria to establish the diagnosis of DRESS (Kardaun et al. 2007). The culprit drug (or culprit drugs), determined according to the French Pharmacovigilance criteria (Moore et al. 1985),(Bégaud et al. 1985), were stopped and patients were regularly evaluated for clinical and laboratorial

parameters, according to the evolution of the disease and response to treatment. Whenever possible, and after informed consent, a 5mm punch diagnostic skin biopsy was performed, under local anaesthesia, in the first days of hospitalization.

The present study included only in-patients fulfilling the referred DRESS criteria who performed skin biopsy.

The following data were evaluated in these patients:

- age and sex;

- culprit drug and latency period between drug intake and onset of DRESS, defined as the date of beginning of the exanthema, fever and/or the other systemic symptoms;

- pattern of cutaneous lesions, defined as an exfoliative erythroderma, a maculopapular generalized exanthema, with or without associated cutaneous lesions, like bullae, pustules, purpura or atypical targets;

- facial edema and mucosal involvement;

- duration of the reaction;

- presence of lymphadenopathy;

- involvement of systemic organs;

- DRESS score, evaluated according to the RegiSCAR criteria (Kardaun et al. 2007)

- presence and intensity of liver cytolysis and/or cholestasis, characterizing cholestasis as more than twice the upper limit of gamma glutamyl transpeptidase (GGT >110 IU/L) and liver cytolysis as more than twice the upper value for alanine aminotransferase (ALT > 70 IU/L), at the highest of all determinations; liver cytolysis was considered moderate (>2x up to 10x the normal, i.e. 71 to 350 IU/L), severe (>10x up to 20x the normal, i.e. 351 to 700 IU/L) and very severe (>20x the upper limit, i.e. > 700 IU/L);

- presence of circulating atypical lymphocytes;

- presence and intensity of eosinophilia, defined as 700 x 10^6 or more eosinophils/L or more than 10% of circulating leukocytes at the highest determination; eosinophilia was considered moderate between 700 and 1499 x 10^6 eosinophils/L or between 10 and 19.9% of all circulating leukocytes and severe eosinophilia if there were ≥1500 x 10^6 eosinophils/L or ≥20% of all circulating leukocytes;

- serologic evidence of recent EBV, CMV or HSV infection or reactivation; and,

- detection of serum DNA for HHV-6, by real time PCR with a commercial kit.

Other causes of fever and exanthema with multi-organ involvement were excluded, like viral hepatitis (serology for HAV, HBV or HCV), systemic infection (culture of blood smears) or an autoimmune disease (antinuclear antibodies and anti-ds-DNA, respectively by IFI and ELISA).

Additionally, we registered the day of evolution of the DRESS when biopsy was performed, the area of the skin where the biopsy was collected as well as the laboratory parameters within 24h of performing skin biopsy, namely eosinophil count and serum ALT values.

D.2.2. Histopathology study

After biopsy, skin fragments were immediately placed on formalin, fixed for 24h, processed for routine light microscopy and stained with haematoxylin-eosin.

Histologic slides were then separately observed by two independent Dermatopathologists with no knowledge of the clinical data of the patients. Different parameters were evaluated and scored between zero and a maximum value of 6, with zero as absent, 2 as moderate, 4 as intense and 6 as very intense. An arithmetic mean of the scores performed by the 2 Dermatopathologists was calculated and used thereafter.

The following epidermal histopathologic parameters were evaluated and scored: alterations of the horny layer (orthokeratosis or parakeratosis), spongiosis, exocytosis of lymphocytes, neutrophils and eosinophils, vacuolar degeneration of the epidermal basal cell layer and keratinocyte necrosis, evaluated as isolated or confluent, basal or suprabasal. Intensity of basal cell vacuolization and keratinocyte necrosis were also evaluated, as a whole, and designated as the epidermal damage score.

In the dermis, the intensity of the inflammatory infiltrate was evaluated at different localizations (perivascular, interstitial, at the dermal-epidermal junction, periadnexal or intravascular) and the different types of the main inflammatory cells (lymphocytes, neutrophils or eosinophils) were considered separately. Furthermore, the intensity of dermal edema and extravasated red blood cells was also evaluated and scored.

After evaluating the intensity of these parameters separately, a further score was calculated for the intensity of the lymphocyte, eosinophil and neutrophil infiltrate, as the sum of their scores in the epidermis, dermis (perivascular, interstitial, at the dermal-epidermal junction and periadnexal) and within the lumen of blood vessels (from 0 to 42), that we designated as the lymphocyte score (Ly score), the eosinophil score (Eos score) and neutrophil score (Neut score).

D.2.3. Statistical correlation of data

Correlation between several of the isolated and combined histopathologic parameters was calculated, namely between the scores of epidermal damage and the inflammatory infiltrate. Moreover, additional correlations were calculated between histological scores and clinical and laboratorial data, namely between scores for epidermal damage and inflammatory infiltration and the general DRESS score, the severity of liver cytolysis (ALT) or cholestasis

(GGT) and serum eosinophilia, both at their highest values and at the time skin biopsy was performed.

A linear relationship between 2 independent variables was evaluated calculating the Pearson's correlation coefficient, the "Pearson's r", where values near '+1' represent a strong direct linear relationship between the 2 variables, the values around 'zero' reflect no correlation and values near '-1' reflect a strong inverse linear relationship between the 2 variables. The coefficient of determination (r^2), which actually measures the strength of the correlation, and the *p*-value for the correlation coefficient were calculated, based on the size of our sample (n=15), and were considered significant when $p \leq 0.05$ or very significant when $p \leq 0.01$.

Moreover, correlation between some histological parameters and the culprit drug, the pattern of the exanthema and the presence, or not, of circulating DNA for HHV-6, was also evaluated, using the student t-test.

D.3. Results I - Clinical characterization of patients with DRESS

Between 2010 and 2013, we studied skin biopsies from 15 patients with a diagnosis of DRESS. In 5 patients there was a DRESS score of 4 or 5, therefore defined as a probable DRESS, and in 10 a score between 6 and 8, therefore defined as a definite case of DRESS.

They were 9 males and 6 females, aged 17 to 87 years' old (mean 53.3 ± 22.3 years), with an older age in females (59.7 years) than in males (48.9 years).

The culprit drug was allopurinol in 8 patients, an anticonvulsant in 5 (3 cases from carbamazepine and 2 from phenytoin) and cotrimoxazole and minocycline, in 1 case each.

In two patients, who were genotyped as HLA-A*31:01 and HLA-B*58:01, DRESS was imputed with high probability, respectively, to carbamazepine and

allopurinol. These two patients had also used amoxicillin during the preliminary symptoms of DRESS (fever, malaise and flu-like or ORL symptoms). Nevertheless, this antibiotic was not considered the culprit drug due to the non-compatible time interval and, also, because another more probably drug was identified, whose responsibility was also supported by the presence of the HLA susceptibility genes.

The mean latency period from the beginning of treatment with the culprit drug to the onset of the DRESS varied between 10 and 42 days (mean 23.5 ± 8.2 days) (Table D.1).

Overall, DRESS manifestations lasted longer than two weeks in all patients and included high fever and a generalized exanthema involving >50% of the body surface area (BSA). Visceral involvement occurred in all 15 patients, in 9 patients affecting more than one internal organ.

Lymphadenopathy, involving more than one area, occurred in 5 patients (Table D.1).

The exanthema initiated shortly after fever and presented as an exfoliative erythroderma in 3 cases and a generalized symmetric erythematous macular and papular eruption in 12. Cutaneous lesions were almost confluent and affected more than 90% of BSA in 3 patients.

Concomitant atypical targets were observed in 2 patients, purpura in 3 and skin fragility in 1. Large flaccid bullae were observed in one patient, who also had severe hepatic cytolysis and kidney disease (pt. 4).

Facial edema occurred in 9 cases (60%), with facial pustules and lip erosions, respectively in 1 and 2 patients (Table D.2), (Fig. B.3 and Figs. D.1-D.4).

Table D.1 - Characteristics of patients with DRESS, including the year of diagnosis, patients' age and sex, culprit drugs and interval between onset of drug intake and onset of the reaction, elements to evaluate the DRESS criteria (fever, exanthema, lymphadenopathy, eosinophilia, atypical lymphocytes, involved organs, duration >15 days, absence of other causes) and calculation the final DRESS Score, according to RegiSCAR criteria (Kardaun et al. 2007)

Pt	Year	Age	Sex	Culprit drug	Interval	Fever	Exanthem	LN	EOS	Atypic Ly	Involved organs	>15 days	Other causes	DRESS Score
1	2010	70	M	carbamazepine* + amoxicillin	42	0	2	1	2	0	1	0	1	7
2	2010	78	F	allopurinol**	21	0	2	0	1	0	2	0	1	6
3	2010	26	M	allopurinol ** + amoxicillin	21	0	2	1	2	1	1	0	1	8
4	2010	47	F	allopurinol**	21	0	2	0	2	0	2	0	1	7
5	2011	68	M	phenytoin	15	0	2	1	1	0	2	0	1	7
6	2011	39	F	carbamazepine	34	0	2	0	2	1	1	0	1	6
7	2011	87	F	phenytoin	21	0	1	0	1	0	1	0	1	4
8	2011	29	M	allopurinol	21	0	2	1	2	0	2	0	1	8
9	2011	67	F	allopurinol**	21	0	2	0	2	0	1	0	1	6
10	2012	78	M	allopurinol**	35	0	2	0	1	0	1	0	1	5
11	2012	63	M	allopurinol**	30	0	2	0	0	0	1	0	1	4
12	2012	25	M	cotrimoxazole	10	0	1	0	2	1	1	0	0	5
13	2012	40	F	carbamazepine	24	0	2	0	0	1	2	0	1	6
14	2013	17	M	minocycline	21	0	2	1	0	0	1	0	0	4
15	2013	66	M	allopurinol	15	0	2	0	1	1	2	0	1	7
		53,3 years	9M/ 6F		23,5 days	15	15	5	12	5	15	14	13	

Scoring for the DRESS criteria:
Fever: -1: absence of fever; - 0: fever (>38,5ºC);
Exanthema: - 2: exanthema involving more >50% of body surface area (BSA) and characteristic of DRESS; - 1: exanthema in the absence of one of the previous characteristics;
LN (Palpable lymph nodes): - 0: absent; - 1: present;
EOS (Eosinophils): - 0: <700x10^6 eosinophils/L or <10% of all leucocytes; - 1: 700-1499 x10^6 eosinophils/L or 10-19.9% of all leukocytes; - 2: >1500 x10^6 eosinophils/L or >20%;
Internal organ involvement: - 0: no systemic organs involved; - 1: one internal organ involved; - 2: more than 1 organ involved.
* This patient was genotyped as HLA-A*31:01; ** These patients were positive for HLA-B*58:01.

The liver was affected in all patients, either with cytolysis (8) cholestasis (1) or both (6). Mean ALT and GGT values were, respectively, 709.3 ± 1208.9 and 170.3 ± 154 IU/L.

In 2 patients (pts 4 and 6), liver cytolysis was very severe, with a transient phase of liver failure (low serum prothrombin in the two patients and both a reduced coagulation factor VII and a borderline factor V in one of them) (pt. 4).

Kidney function was transiently impaired in 5 patients. Another patient, with previous proteinuria (pt. 4), progressed to a severe glomerulonephritis that is now under immunosuppressive drugs, while waiting kidney transplantation. Five of the 6 cases with kidney involvement were induced by allopurinol.

Digestive tract was affected in 2 patients, with abdominal pain or diarrhoea (Table D.2).

Atypical circulating lymphocytes were observed in 5 patients. One patient had severe leukopenia (1900 x 10^6 leucocytes/L) (pt 10). Leucocytosis with 2% circulating myelocytes and metamyelocytes was observed in 2 patients (pt 6 and 9).

A high eosinophil count occurred in 12 patients, 7 of whom with severe eosinophilia (>1500 x 10^6 cells /L or > 20% of circulating leucocytes). Eosinophilia was not always present at admission. The maximum eosinophil count was observed only after day 10, in two patients (pts 3 and 8).

Circulating DNA for HHV-6 was present in 3 out of the 6 patients tested during the 1st or 2nd week of hospitalization (Table D.1 and D.2).

Table D.2 - Clinical and laboratorial characteristics of DRESS cases, including muco-cutaneous manifestations and results of liver function tests, eosinophil count and presence of circulating viral DNA from HHV-6

Pt	Age	Sex	Culprit drug	Exan thema	facial edema	other skin manifestations	Int. organs involved	ALT	GGT	EOS max		HHV-6 DNA
										%	value	
1	70	M	CBZ+ AMX	ERYT	No		L1	79	77	33,0%	3916	n.t.
2	78	F	ALP	MPE	Yes	oral erosions BSA>90%	L1, Kd	120	18	14,2%	1100	n.t.
3	26	M	ALP+ AMX	ERYT	Yes	purpura	L3	730	196	19,1%	5800	n.t.
4	47	F	ALP	MPE	Yes	BSA>90% lip erosions flaccid bullae	L3*, Kd*	3033	331	16,5%	1800	n.t.*
5	68	M	PHY	MPE	No	atypical targets	L1, Dig	90	89	8,2%	775	n.t.*
6	39	F	CBZ	MPE	Yes		L3, Hemat	4059	556	20,0%	4800	Pos
7	87	F	PHY	MPE	No	purpura	L2	33	281	8,6%	940	n.t.
8	29	M	ALP	MPE	Yes	facial pustules purpura skin fragility	L3, Kd	1155	360	18,0%	3600	Pos
9	67	F	ALP	MPE	Yes	atypical targets	L1, Kd, Hemat	98	53	18,3%	3500	Neg
10	78	M	ALP	ERYT	No		L1, Hemat	105	88	13,9%	640	Pos
11	63	M	ALP	MPE	Yes		L3	231	214	4,6%	350	n.t.
12	25	M	CTX	MPE	Yes		L1	96	38	12,0%	1764	n.t.
13	40	F	CBZ	MPE	No		L3, Kd, Dig	169	171	4,5%	301	Neg
14	17	M	MNC	MPE	Yes	purpura	L1	562	59	6,8%	433	n.t.
15	66	M	ALP	MPE	No	BSA>90%	L1, Kd	79	24	13,9%	840	Neg
mean								709.3± 1208.9	170.3± 154.9		2037± 1803.4	

ALT – alanine aminotransferase (normal < 35 IU/L); GGT – gamma glutamyltranspeptidase (normal < 55 IU/L);
EOS – serum eosinophils: % of circulating white blood cells and absolute value;
CBZ – carbamazepine; AMX- amoxicillin; ALP-allopurinol; PHY – phenytoin; CTX – cotrimoxazole; MNC – minocycline;
ERYT – exfoliative erythroderma; MPE – macular and papular exanthema;
BSA – body surface area;
L1 – liver cytolysis; L2 – liver cholestasis; L3-liver cytolysis + cholestasis;
Kd – kidney involvement; Dig – digestive tract involvement; Hemat – hematologic alterations;
n.t. – not tested
*HHV-6 DNA was not tested but these patients had high IgG (not IgM) antibodies for HHV-6

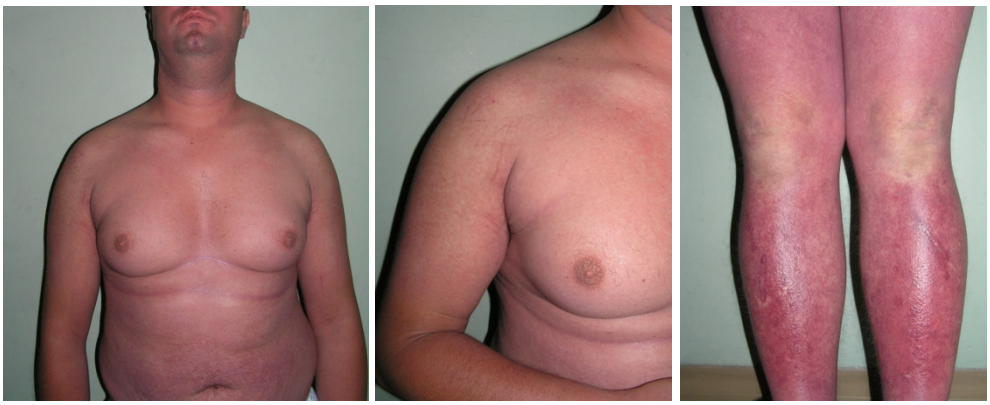


Figure D.1 - Erythroderma with purpuric areas, mainly in the lower limbs, in a DRESS induced by allopurinol (pt. 3).

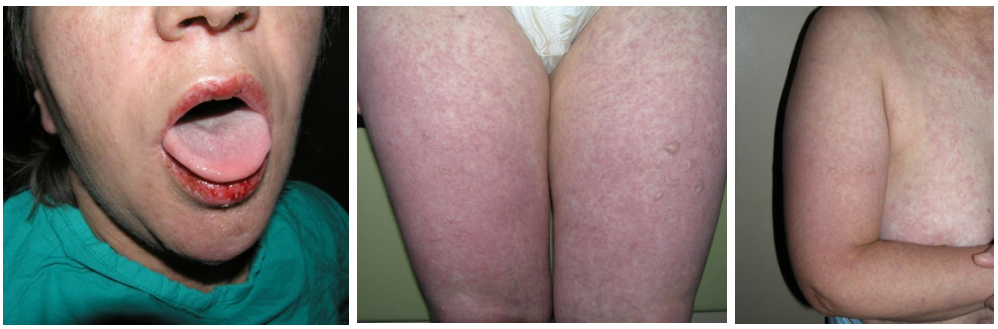


Figure D.2 – Generalized symmetric macular and papular exanthema with flaccid bullae on the chin, arms and thighs, and erosions of the semi-mucosae of the lips with no internal involvement of the oral mucosae. Pt. 4 with a severe and long lasting allopurinol induced DRESS.

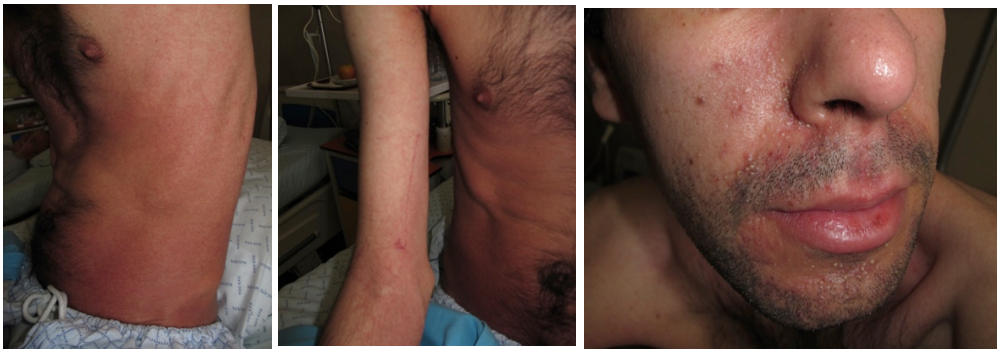


Figure D.3 - Maculo-papular exanthema with purpura, skin fragility and facial pustules in pt. 8 with an allopurinol induced DRESS.

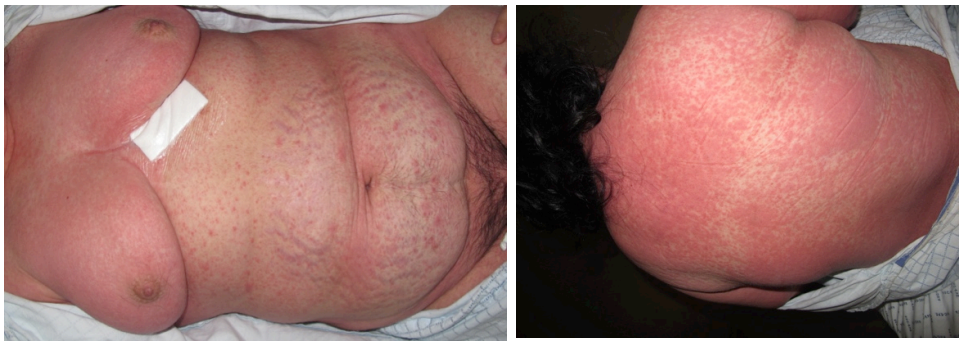


Figure D.4 – Carbamazepine-induced DRESS with symmetric generalized maculo-papular exanthema with isolated lesions, resembling atypical targets in some areas (pt. 13).

The culprit drug was stopped on admission and all patients received supportive treatment and systemic corticosteroids (0.5 to 0.75 mg/Kg of body weight/day), with a very slow tapering and drug adjustments, according to clinical improvement or relapses.

All patients survived the DRESS, but patient 5 had a fatal outcome 7 months after DRESS resolution, due to the brain tumour that had motivated the use of phenytoin.

Patient 4, who required prolonged immunosuppressive therapy, had a severe herpes zoster affecting more than one dermatome and required i.v. acyclovir, but she recovered completely with no sequelae. No cases of autoimmunity were observed in the 6 months following suspension of corticosteroids.

D.3.1. Characterization of patients at the time of biopsy

Skin biopsies of the exanthema were performed on the most affected skin area with recent lesions (arms, thighs, abdomen or trunk), between the 5th and 35th

day of evolution of the DRESS (mean 12.9 ± 7.1 days), with 10 cases taken before day 15 and 4 taken at day 15 or 16, considering day one as the onset of fever, rash or other symptoms related to DRESS (Table D.3).

Hepatic liver enzymes were normal in 3 patients and 12 showed cytolysis with ALT varying from 63 to 3556 (mean 446.9 ± 900.3). Patients 6 and 8 had a significant liver cytolysis (ALT = 3556 and 982 IU/L) at the time of skin biopsy.

The eosinophil count varied from 34 to 5800 x 10^6 cells /L (mean 1243.7 ± 1695.4). Severe eosinophilia (above 3000 cells x 10^6/L) and moderate eosinophilia (between 700 and 1500 cells x 10^6/L) were present, respectively, in 3 and 5 patients (Table D.3).

Table D.3 – Characteristics of the patients at the time skin biopsy was taken, namely the day of the evolution of DRESS, the local were the fragment was collected and the results of liver cytolysis evaluated by ALT value and total eosinophil count, determined within 24 h of performing skin biopsy

Pt	Age	Sex	Culprit drug	Histology number	Day of biopsy	Local of biopsy	EOS count	ALT
1	70	M	CBZ +AMX	68655	35	arm	740	71
2	78	F	ALP	70838	15	arm	1100	80
3	26	M	ALP+AMX	69693	12	thigh	5800	104
4	47	F	ALP	69688	13	thigh	1100	461
5	68	M	PHY	71664	8	abdomen	700	69
6	39	F	CBZ	72717	16	arm	48	3556
7	87	F	PHY	72451	13	arm	900	34
8	29	M	ALP	72328	11	thigh	3600	982
9	67	F	ALP	74252	6	abdomen	3500	98
10	78	M	ALP	74984	16	arm	60	32
11	63	M	ALP	75917	9	trunk	303	221
12	25	M	CTX	75113	9	trunk	34	35
13	40	F	CBZ	74569	5	trunk	300	335
14	17	M	MNC	77164	9	thigh	120	562
15	66	M	ALP	76122	15	abdomen	350	63
Mean ± STD					12.9 ± 7.1		1243 ± 1695.4	446.9 ± 900.3

EOS – eosinophils; ALT – alanine aminotransferase (IU/L – normal <35 IU/L); CBZ – carbamazepine; AMX-amoxicillin; ALP-allopurinol; PHY – phenytoin; CTX – cotrimoxazole; MNC – minocycline; STD – standard deviation

D.4. Results II – Histopathology of the exanthema in DRESS

The histopathologic changes observed were highly variable, both on the type of dermal or epidermal changes and in their intensity, often with little correlation between dermal and epidermal findings.

The most frequently observed epidermal changes on histopathology were mild spongiosis (12 pts) with lymphocyte exocytosis (13 pts), vacuolar degeneration of the basal layer (14 pts) and keratinocyte necrosis (12 pts), mostly under an orthokeratotic horny layer (14 pts).

Dermal edema was observed in 13 patients with a perivascular and interstitial infiltrate of lymphocytes (all 15 pts), eosinophils (11 pts) or neutrophils (2 pts), mostly around blood vessels (15 pts), near the dermal-epidermal junction (13 pts) and surrounding skin adnexa (3 pts). Intravascular lymphocytes and neutrophils were observed in 7 patients each and extravasation of red blood cells was documented in 12 (Fig. D.5a-h).

Epidermal and dermal changes were associated in distinct combinations and could be grouped on some general histologic patterns, although not always very clearly defined: - an eczematous reaction, with spongiosis and exocytosis, in 5 patients; - an erythema multiforme-like pattern, with intense basal cell vacuolization and keratinocyte necrosis, in 3 patients, involving almost the whole thickness of the epidermis in one; - a band-like lichenoid infiltrate, with vacuolization of basal epidermal cells and spotty epidermal necrosis, observed in 2 patients; - a predominantly dermal inflammation and edema or a pseudolymphoma-like pattern with a dense band-like lymphocyte infiltrate with atypical cells in the upper dermis, in one single patient; - mixed and relatively undefined patterns were present in the other 4 patients.

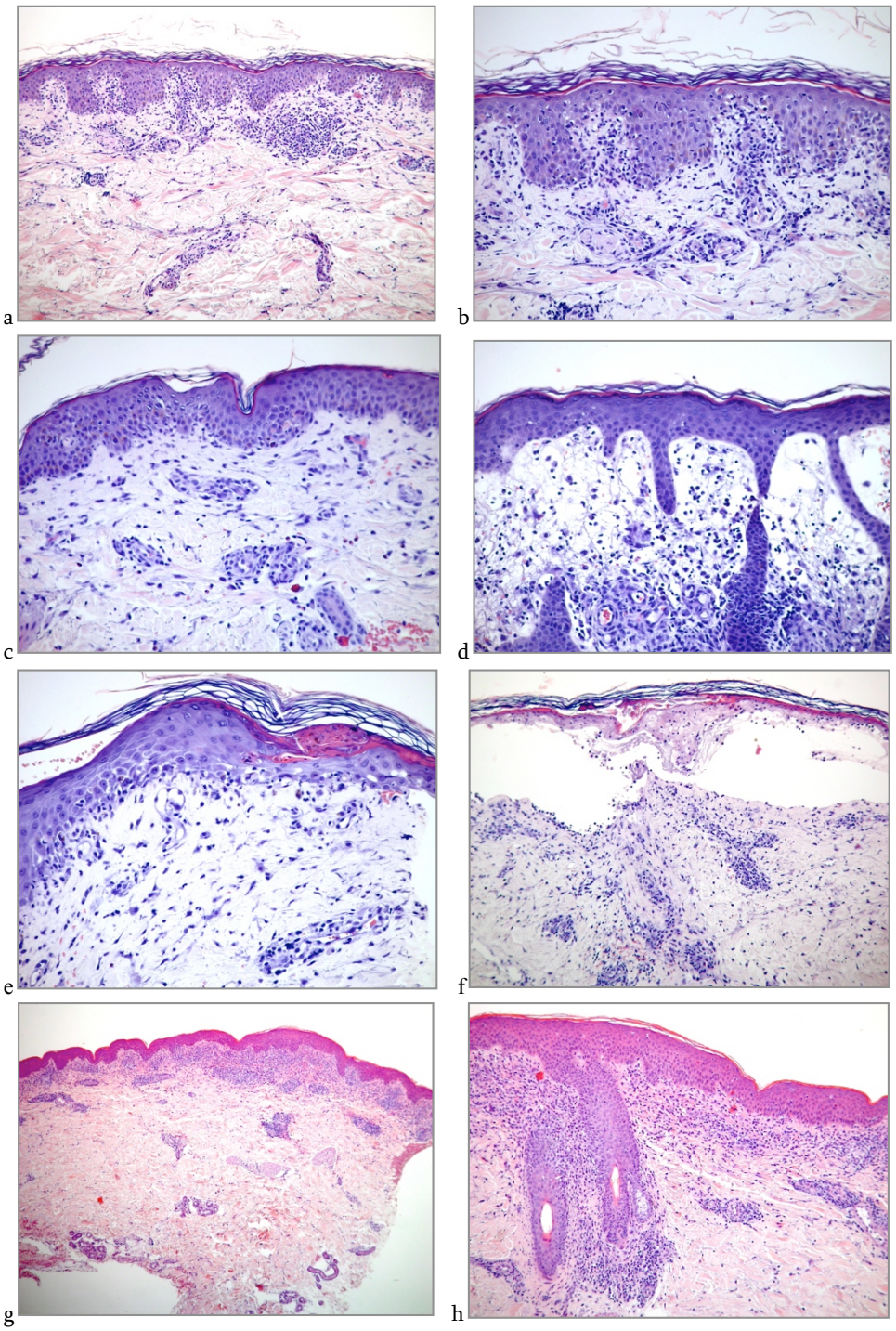


Figure D.5 - Main histologic patterns observed in DRESS. Predominance of exocytosis and epidermal vacuolization in pt.9 (a,b), a predominant dermal perivascular infiltration with necrotic keratinocytes in pt. 5 (c), a prominent dermal edema with scarce epidermal involvement in pt 3 (d), an erythema multiforme type with intense basal cell vacuolization and even full thickness epidermal necrosis in spotty areas in pt. 4 (e,f) and a pseudolymphoma-like pattern with a dense band-like lymphocyte infiltrate with atypical cells localized in the upper dermis and perifollicular in pt. 6 (g,h).

D.4.1. Histopathology: epidermal changes

Epidermal horny layer was mostly orthokeratotic (14 pts), compact in 2 cases. Parakerathosis was observed in some areas of the biopsies in 5 patients. It was mostly focal parakerathosis (2 pts) or parakerathosis in mounds (2 pts) (Fig. D.6c).

Spongiosis, present in 12 patients, was highly variable, with a mean score of 0.93 ± 0.94, and was mostly prominent in patient 7 (score 3).

Thirteen patients exhibited a discrete to moderate lymphocyte exocytosis (mean score 1.87 ± 1.25), particularly evident in patients 1, 7 and 15 (Fig. D.5a-c; D.6e,f), whereas eosinophil exocytosis occurred only in 1 patient (Table D.4).

Neutrophil exocytosis was observed in 2 cases, forming a subcorneal spongiform pustule similar to those described in AGEP, in patient 1 (Fig. D.6a,b). This patient, biopsied very late during the course of the CADR (day 35), had an exfoliative erythroderma with no macroscopic pustules, whereas patient 8 who had facial pustules (Fig. D.3), had no neutrophil epidermal infiltration or pustule formation in a skin biopsy collected from the thigh.

In 14 of the 15 patients there was vacuolar degeneration of basal keratinocytes (mean score 2.13 ± 1.42), in 3 cases with moderate to severe intensity (Fig. D.6e-g).

Necrotic keratinocytes occurred in 12 patients, but with a varying intensity (mean 1.70 ± 1.44). They were observed, particularly, in suprabasal layers and were mostly isolated necrotic keratinocytes, although in 4 patients confluent areas of necrosis were observed (Fig. D.6c-h). Keratinocyte necrosis was particularly intense, with necrosis of almost the full thickness of the epidermis in some areas, in patient 4 (Fig. D.5f and D.6h), who had a maculo-papular exanthema with flaccid bullae (Fig. D.2), and severe hepatic cytolysis and kidney damage (Table D.2).

The evaluation of the association of hydropic degeneration of the basal cell epidermal layer and keratinocyte necrosis, quantified separately as the

"epidermal damage score" and considered as a marker of epidermal aggression, varied between zero and the maximal value of 6 (mean 2.30 ± 1.66).

Table D.4 - Epidermal changes observed in skin biopsies, with the mean scores of independent observations by 2 Dermatopathologists, and calculation of an epidermal damage score (combination of the intensity of vacuolization of basal layer and keratinocyte necrosis)

Pt	Horny layer		Spongiosis	Exocytosis			Keratinocyte necrosis					Basal vacuolation	Epiderm damage score
	Ortho keratosis	Para keratosis		Ly	Neut	Eos	Isolate	Confluent	Basal	Supra basal	Overall necrosis		
1	0	3++	1	3,5	2,5	0	1	0	1	0,5	1	1	1,5
2	2	0,5**	0	0,5	0	0	2	2,5	1	2	2,5	4	4
3	2	0,5***	1	2	0	0	1	0	0	1	1	1	1
4	2	0	2	2,5	0	0	3	5	3	4	5	5	6
5	2	0	2	2	0	2	3,5	0,5	1,5	3,5	2	2	2
6	2*	0	1	2,5	0	0	2,5	0	1	2	2	2	2
7	2+	0	3	3,5	0	0	1	0	1	0	1	4	3
8	2	0	0	0,5	0	0	0,5	0	0	0,5	0,5	1	1
9	2	0	0	3	0	0	3	0	1,5	4	3,5	3	4,5
10	2	2**	0	2	0,5	0	2	1	2	4	3	2	3
11	0,5*	4***	1,5	1,5	0	0	0	0	0	0	0	0,5	0
12	2	0	0	1	0	0	2	0	0	2	1,5	1,5	2
13	2	0	1	0	0	0	0	0	0	0	0	2	1,5
14	2	0	0	0	0	0	0	0	0	0	0	0	0
15	2	0	1,5	3,5	0	0	3	0	1,5	2	2,5	3	3
mean score	1.80	0.67	0.93 ± 0.94	1,87± 1.25	0.20± 0.65	0.13± 0.52	1.63± 1.22	0.60± 1.39	0.90± 0.91	1.70± 1.57	1.70± 1.44	2.13± 1.42	2.30± 1.66
Total +	14	5	12	13	2	1	12	4	10	11	12	14	13

* compact horny layer; + epidermal atrophy; ++parakeratosis with neutrophils forming a subcorneal pustule, as usually observed in AGEP; ** focal parakeratosis; *** parakeratosis in mounds; Ly - lymphocytes; Neut – neutrophils; Eos- eosinophils

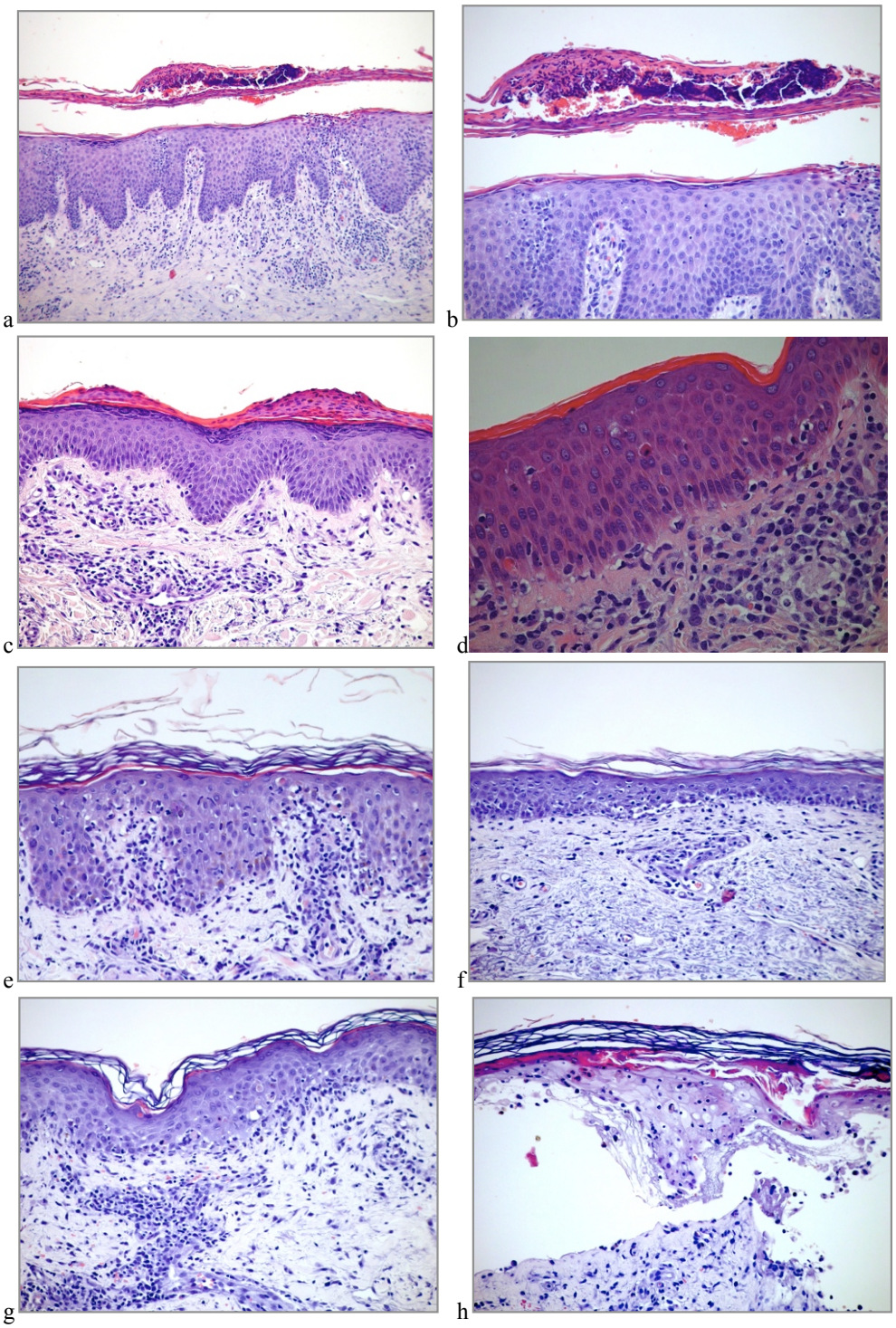


Figure D.6 – Epidermal changes. A subcorneal epidermal spongiform pustule in pt.1 (a,b) and parakerathosis in mounds in pt.11(c), keratinocyte necrosis/apoptosis in basal and mostly suprabasal layers (d,e) associated with intense exocytosis in pt. 9 (e) and intense vacuolization of basal cells, forming suprabasal clefts under an atrophic epidermis in pt. 7 (f) or more intense vacuolization with areas of necrosis of the whole epidermal thickness in pt.4 (g,h).

D.4.2. Histopathology: dermal changes

Dermal edema was observed in 13 out of the 15 patients studied (mean 1.47 ± 1.34). It was particularly intense in patient 3, with erythroderma and facial edema, who had the highest eosinophil count but very discrete liver cytolysis (Figs. D.5.d, D.7.a).

All biopsies showed a perivascular lymphocyte infiltrate, with a variable intensity, with no signs of vasculitis but, often, with turgescent endothelial cells (Fig. D.7a,g-h). Extravascular red blood cells occurred in 12 patients, particularly in patients 3 and 8, who had visible purpura (Table D.4, Fig. D.7f).

Dermal lymphocyte infiltrate was observed mainly in the upper dermis, most intense in the perivascular compartment (mean score 2.93 ± 0.92), near the dermal-epidermal junction (mean score 2.07 ± 1.12) and in the interstitium of the dermis (mean score 1.63 ± 0.94) (Fig. D.7a-f).

The lymphocyte infiltrate was more intense around the adnexa, particularly in a perifollicular distribution (Fig. D.7e), in patients 6 and 10, two of the 3 who showed circulating HHV-6 DNA during the acute stage of the DRESS (Table D.2 and D.5).

A dense infiltrate with atypical lymphocytes, characteristic of a pseudo-lymphomatous reaction (Fig. D.7b-e), occurred in patient 6, with a carbamazepine induced erythemato-papular exanthema with confluent lesions. This patient had severe eosinophilia early at admission (day 4), but a late onset of liver cytolysis, which progressed to incipient liver failure. She also showed circulating atypical lymphocytes, only by day 16, when skin biopsy was performed. Both this patient and patient 4 had a band-like distribution of the infiltrate at the dermal-epidermal junction, but a very intense vacuolization of basal keratinocytes occurred only in patient 4 (Table D4 and D5).

Eosinophils were present in 11 of the 15 patients, sometimes with a significant intensity, when considered the whole esosinophil score (mean score 2.63 ± 2.39 in the maximum of 42). They were observed mainly in the interstitium of the dermis (11 pts, mean score 1.20 ± 1.08) or intermingled with the perivascular

lymphocyte infiltrate (9 pts, mean score 1.07 ± 1.38) and seldom around adnexa (2 pts), at the dermal-epidermal junction (1 pt) or inside blood vessels (4 pts.).

Neutrophils were observed in 10 biopsies (mean overall score of 0.86 ± 1.01). They were very scarce in the perivascular or interstitial inflammatory infiltrate, but more frequent within the lumen of blood vessels. No neutrophils were observed either at the dermal-epidermal junction or the periadnexal area.

Table D.5 - Dermal changes observed in skin biopsies, with the mean scores of the independent observations by 2 Dermatopathologists, and calculation of the scores for dermal-epidermal infiltration of lymphocytes (Ly score), neutrophils (Neut score) and eosinophils (Eos score)

Pt	Derm edema	Inflammatory infiltrate																Extra vasc RBC	Ly Score	Neut score	Eos Score
		Perivascular			Interstitial			Periadnexal			DE junction			Intravascular							
		Ly	Neu	Eos	Ly	Neu	Eos	Ly	Neu	Eos	Ly	Neu	Eos	RBC	Ly	Neu	Eos				
1	0,5	3	1	2	2*	0,5	2	0	0	0	3	0	0	1	0	1	1	0	11,5	2,5	5
2	1	2	0	3	2	0	2,5	0	0	0	3	0	0	2	0,5	0	0	0,5	8	0	5,5
3	5	4	0	0,5	1,5	0	0	0	0	0	2	0	0	4	0	1	0	5	9,5	1	0,5
4	2	2,5	0,5	0,5	1	0	1,5	0	0	0	3,5**	0	0	1,5	0	0	0,5	1,5	9,5	0,5	2,5
5	1,5	2	0	1	1	0	3	0	0	0	1,5	0	0	3	0,5	0,5	0	1	7	0,5	4
6	0,5	3,5*	0	0	4	0,5	0,5	4	0	1	3***	0	0	1,5	1	0	0	3	18	0,5	1,5
7	3	2	0,5	0	1,5	0	2	0	0	0	2,5	0	0	1,5	1,5	0,5	0	2	11	1	2
8	0,5	4	0	4	0,5	0	3	0	0	0	1,5	0	0	1,5	0	0	0	4	6,5	0	7
9	1	4	1	0	1,5	0	0	0	0	0	3,5	0	0	2	0	2	0	0,5	12	3	0
10	0,5	4	0	3,5	1,5	0	1	2	0	2	2	0	1	1,5	0	0	0	2	11,5	0	6,5
11	2	3	0	0	2	0	0	0	0	0	0	0	0	1,5	0	0	0	0	6,5	0	0
12	0	2	0,5	1	1,5	0	0,5	0	0	0	1,5	0	0	0,5	1	0	0	2	7	0,5	1,5
13	2,5	2	0	0	1	0	1	0,5	0	0	1	0	0	1	1	1	0,5	1,5	5,5	1	1,5
14	0	4	0	0	0,5	0	0	0	0	0	0	0	0	3	0,5	0	0	0,5	5	0	0
15	2	2	0,5	0,5	3	0	1	0	0	0	3	0	0	2	0	2	0,5	0	11,5	2,5	2
mean score	1.47 ± 1.34	2,93 ± 0.92	0.27 ± 0.37	1.07 ± 1.38	1.63 ± 0.94	0.07 ± 0.17	1.2 ± 1.08	0.43 ± 1.12	0	0.2 ± 0.56	2.07 ± 1.12	0	0.07 ± 0.26	1.83 ± 0.90	0.4 ± 0.51	0.53 ± 0.72	0,17 ± 0.31	1.57 ± 1.50	9.33 ± 3.40	0.86 ± 1.01	2.63 ± 2.39
Total +	13	15	6	9	15	2	11	3	0	2	13	0	1	15	7	7	4	12	15	10	12

*pseudo-lymphoma-like intense lymphoid infiltrate with atypical cells; **poor cell interface dermatitis ***band-like lichenoid distribution of the lymphoid infiltrate

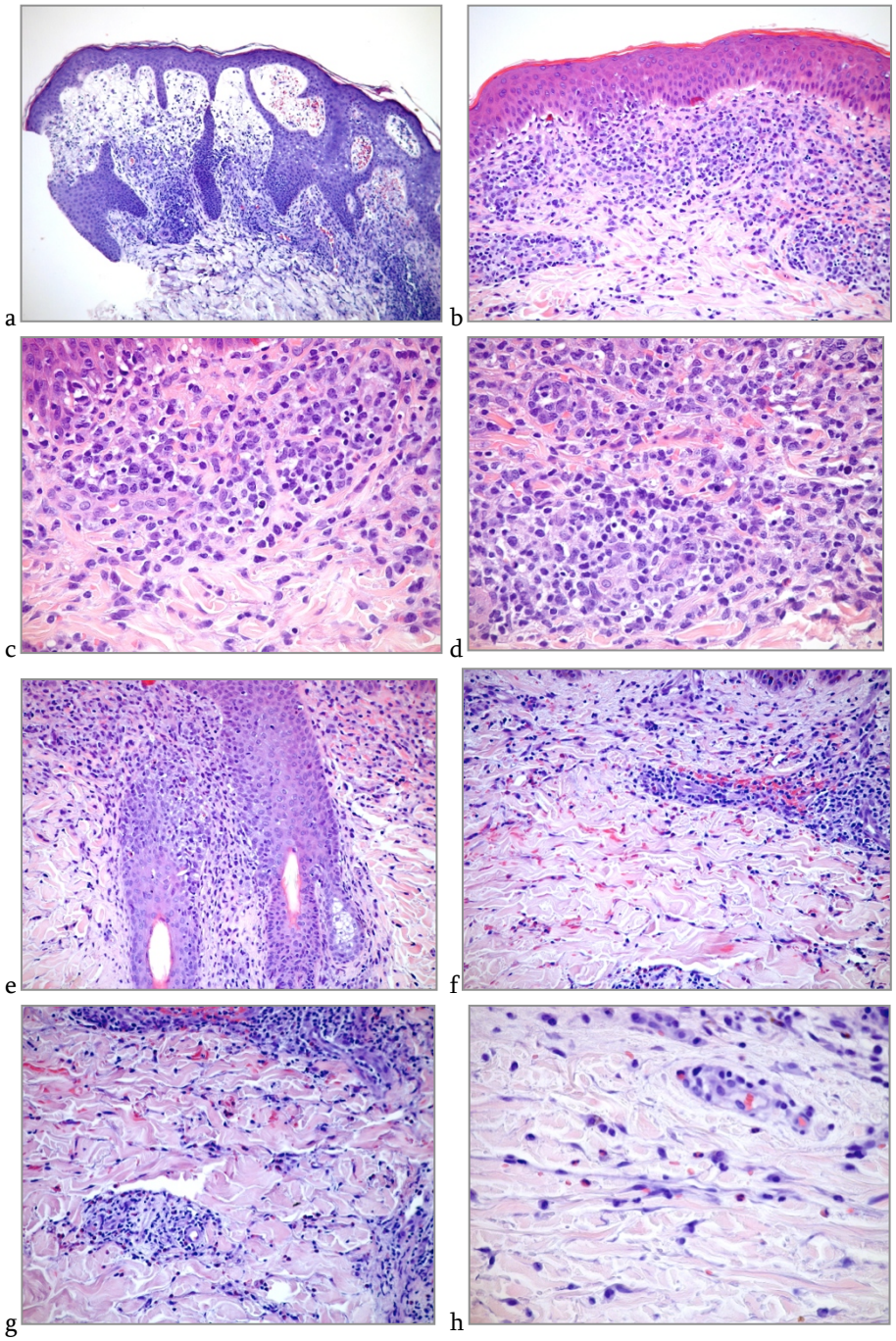
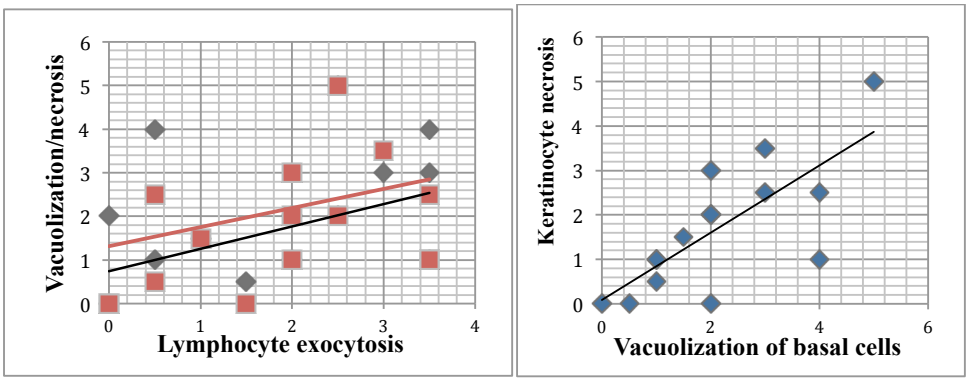


Figure D.7 – Dermal changes. Intense dermal edema and a dense perivascular infiltrate with vasodilation, turgescent endothelial cells and intravascular RBC in pt.3 (a). Dense lymphocyte infiltrate in the upper dermis, dermal-epidermal junction and periadnexal (b-e) with atypical lymphocytes with large and pleomorphic nuclei and rare mitotic figures (c,d) in pt. 6 with a pseudolymphoma like pattern. Perivascular infiltrate with intravascular and extravasated RBC in pt. 8 (f) and eosinophils within the inflammatory infiltrate in pt. 8 (g) or in the interstitium of the dermis in pt. 5 (h)

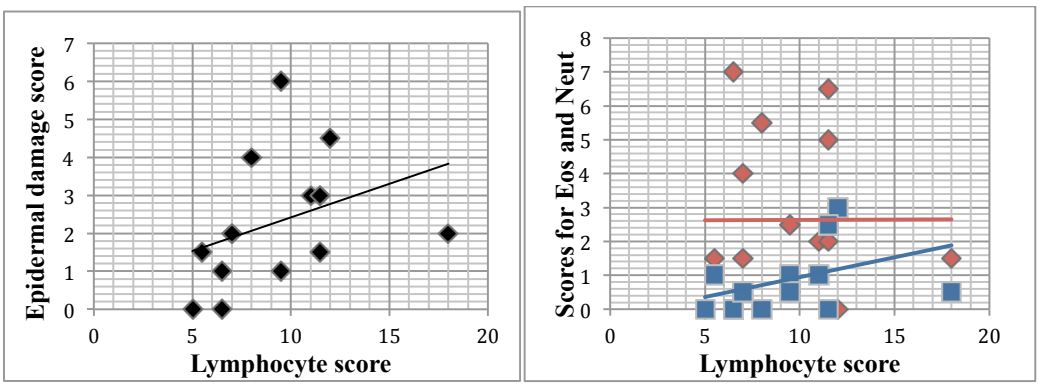
D.4.3. Correlation between different histopathologic changes

There was a weak positive correlation between the intensity of lymphocyte exocytosis and vacuolization at the basal layer and keratinocyte necrosis (respectively, r=0.38 and 0.44) (Graph. D.1), and a moderately positive correlation between vacuolization of basal cells and keratinocyte necrosis (r=0.75) (Graph. D.1). Patient 7, with a phenytoin-induced DRESS whose biopsy was performed at day 13, was an exception, showing the most intense spongiosis (score 3) and an intense vacuolization of the basal layer (score 4) and almost no necrotic keratinocytes (score 1) (Table D.4).



Graphic D.1 Correlation between lymphocyte exocytosis and basal cell vacuolization (*r*=0.38) (■) and keratinocyte necrosis (*r*=0.44) (◆) and between basal cell vacuolization and keratinocyte necrosis (*r*=0.75)(◆).

The sum of the scores for lymphocyte infiltration at dermis and epidermis, the lymphocyte score (Ly score), varied between 5 and 18 (mean 9.33 ± 3.40) (Table D.5). Correlation between the Ly score and degree of keratinocyte necrosis or vacuolization of basal cells, globally referred as the epidermal damage score, was weak (r=0.37) and with important discordant results (Graph. D.2). Patient 6 had the highest Ly score (18) and little keratinocyte damage (score 2), whereas patients 2 and 4 with the highest epidermal damage had a Ly score near the mean, respectively 8 and 9.5.



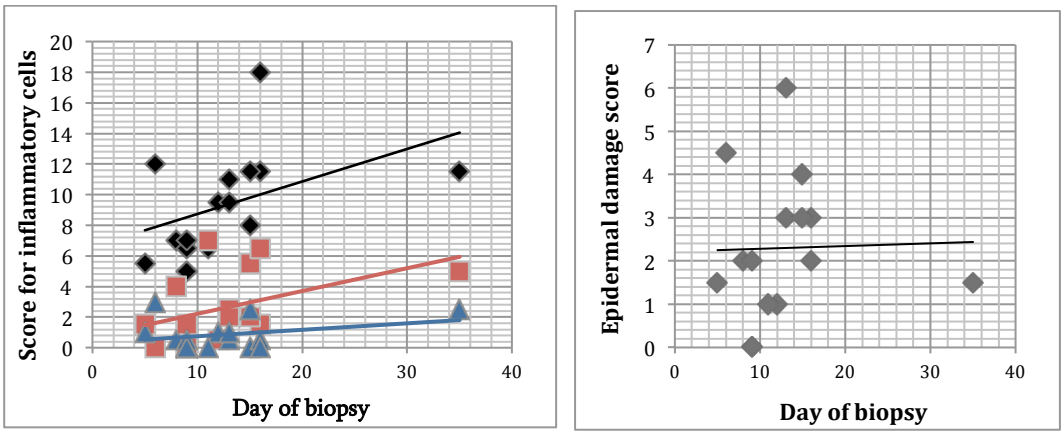
Graphic. D. 2 Correlation between the lymphocyte score and the epidermal damage score (◆) (r=0.37) (left) and the eosinophil score (◆) (r=0) and the neutrophil scores (■) (r=0.39) (right).

The eosinophil score (Eos score) varied between 0 and 7 (mean 2.63 ± 2.39) and was usually more intense than the neutrophil score that varied between 0 and 3 (mean 0.86 ± 1.01). The eosinophil or neutrophil scores were not correlated with the degree of epidermal damage (r=0.17 and r=0.27, respectively) (Table D.5),(Graph. D.2).

Also, there was no correlation between the intensity of the lymphocyte infiltrate and either the neutrophil or eosinophil score (r=0 and r=0.39, respectively) (Graph. D.2.b).

D.4.4. Correlation between histopathology and clinical and laboratorial parameters

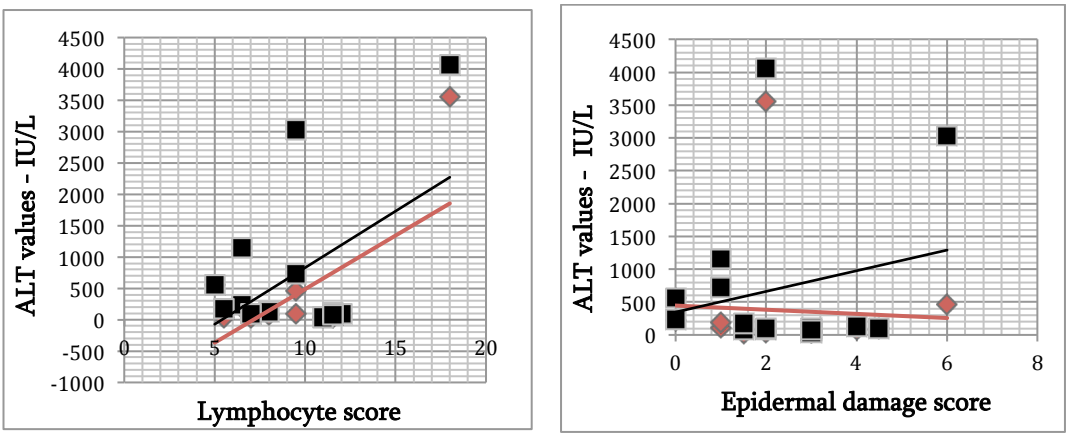
There was a weak, though statistically significant positive correlation, between the day of evolution of the DRESS when skin biopsy was performed and the intensity of the lymphocyte and the eosinophil infiltrate (Ly score and Eos score) (both with r=0.44; p=0.050), whereas there was no correlation between day of biopsy and the neutrophil score (r=0.3; p>0.05) or the epidermal damage score (r=0.03) (Table D.7, Graph. D.3).



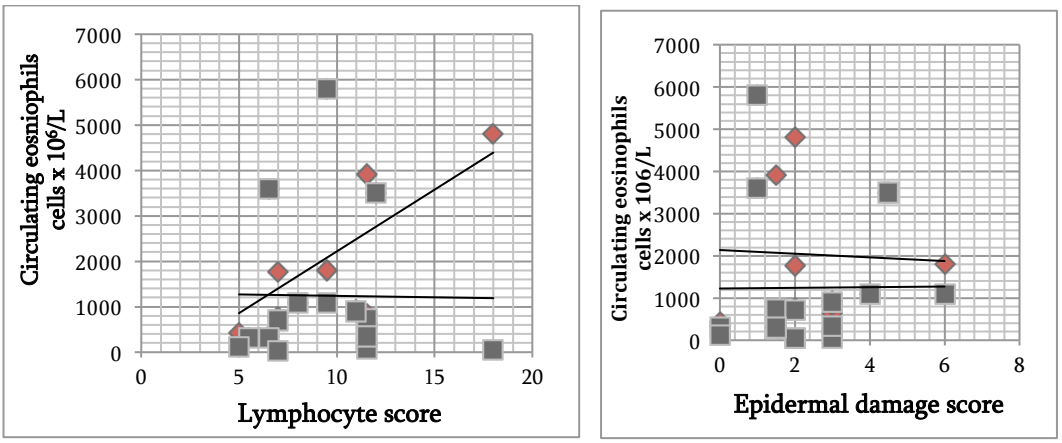
Graphic D.3 – Correlation between the day of evolution of DRESS when biopsy was performed and the intensity of the inflammatory infiltrate (left), considering separately lymphocytes (◆; r=0.44), eosinophils (■; r=0.44) and neutrophils (▲; r=0.3), and the score for epidermal damage (◆; r=0.03) (right).

We tried to establish linear correlations between histology (epidermal damage score, and lymphocyte, neutrophil and eosinophil scores), and clinical parameters, namely the DRESS score, the severity of liver damage, either cytolysis or cholestasis, and eosinophilia (Table D.6 and D7).

We observed a moderate positive and statistically significant correlation between the intensity of the lymphocyte infiltrate and liver cytolysis, at its highest value (r=0.51; $p \leq 0.05$) and, particularly, for the ALT value skin at the moment of performing the biopsy (r=0.65; $p \leq 0.01$). Nevertheless, no correlation was observed between keratinocyte vacuolization, keratinocyte necrosis or the epidermal damage score and the laboratory parameters of liver aggression, neither between eosinophil or neutrophil score and liver aggression (Graph. D.4).

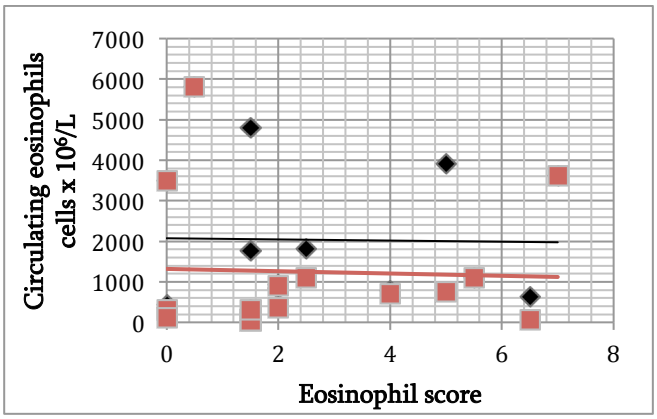


Graphic D.4 - Correlation between the lympocyte score (left) or the epidermal damage score (right) and the degree of liver cytolysis, evaluated by ALT (IU/L) at its highest value during the course of DRESS (■) and at the time of biopsy (◆). A moderate and significant correlation is shown between Ly score and maximum liver cytolysis (r=0.51; p≤0.05) and, particularly, a highly significant correlation is shown with hepatic cytolysis determined at the time skin biopsy was performed (*r*=0.65; p≤0.01). No correlation was shown between the epidermal damage score and parameters of hepatotoxicity.



Graphic D.5 - Correlation between the lympocyte score (left) or the epidermal damage score (right) and the value of circulating eosinophils at the highest value (■) and the time of biopsy (◆). A moderate but significant correlation between the Ly score and the maximum eosinophil count (r=0.51; p≤0.05) was documented, but there was no correlation between the epidermal damage score and the intensity of inflammatory cells invading the dermis / dermal-epidermal junction.

The lymphocyte score also correlated positively with the maximum eosinophil count (r=0.51; p≤0.05), but there was no correlation between the epidermal damage score and eosinophilia (Graph D.5). Interestingly, no correlation was found between the cutaneous eosinophil score (Eos score) and the absolute value of circulating eosinophils, either at its maximum value or within the 24 hour of performing skin biopsy (respectively, r=-0.02 and r=-0.04) (Table D.6 and Graph. D.6). As an example, patients 3 and 9 with allopurinol-induced DRESS, who had the highest circulating eosinophil counts (respectively 5800 and 3500), had no eosinophils in the infiltrate or eosinophils were very scarce in their skin biopsies. On the contrary, patient 10, with a DRESS also induced by allopurinol, had one of the highest Eos score in the skin biopsy (6.5) and no eosinophilia during the course of the DRESS.



Graphic D.6. Correlation between skin eosinophil score and the number of circulating eosinophils (cells x 10^6 /L) at its maximum value (◆; r=-0.02) and at the day of skin biopsy (■; r=-0.04).

No statistically significant and only very weak correlations were observed between the other parameters evaluated, namely between histopathologic parameters and the DRESS score or the intensity of hepatic cholestasis, with most "r" values being near zero, meaning no correlation at all (Table D.7).

Table D.6 – Correlation between the degree of epidermal damage, lymphocyte, eosinophil or neutrophil infiltrate and the culprit drug, DRESS score, circulating eosinophils, liver damage, including cytolysis (ALT) both at the time of biopsy and at its maximum value

	Epiderm damage score	Ly score	Eos score	Neut Score	Day of biopsy	Culprit drug	DRESS score	Circulating eosinophils		ALT at biopsy	Liver damage (maximum value)	
								Maxim value	Time biopsy		ALT	GGT
1	1,5	11,5	5	2,5	35	CBZ	7	3916	740	71	79	77
2	4	8	5,5	0	15	ALP	6	1100	1100	80	120	18
3	1	9,5	0,5	1	12	ALP	8	5800	5800	104	730	196
4	6	9,5	2,5	0,5	13	ALP	7	1800	1100	461	3033	331
5	2	7	4	0,5	8	PHY	7	775	700	69	90	89
6*	2	18	1,5	0,5	16	CBZ	6	4800	48	3559	4059	506
7	3	11	2	1	13	PHY	4	940	900	34	33	281
8*	1	6,5	7	0	11	ALP	8	3600	3600	194	1155	360
9	4,5	12	0	3	6	ALP	6	3500	3500	98	98	53
10*	3	11,5	6,5	0	16	ALP	5	640	60	35	105	88
11	0	6,5	0	0	9	ALP	4	350	303	221	231	214
12	2	7	1,5	0,5	9	CTX	5	1764	34	32	96	38
13	1,5	5,5	1,5	1	5	CBZ	6	301	300	29	169	171
14	0	5	0	0	9	MNC	4	433	120	562	562	59
15	3	11,5	2	2,5	15	ALP	7	840	350	63	79	24
mean	2,30	9,33	2,63	0.8			5.9	2037.3	1243.7	374.1	709.3	167.0

* In patients 6, 8 and 10 DNA for HHV-6 was detected in the blood during the 1st week of the DRESS

Table D.7 – Linear correlation between histopathology, clinical and laboratory parameters, with the calculation of the linear correlation coefficient, Pearson's r with values between -1 (inverse correlation), zero (no correlation) up to +1 (direct positive correlation) and its level of significance (*p*)

Correlation coefficient (Pearson's r)	Day of biopsy	DRESS score	Circulating eosinophils		ALT at biopsy	Liver damage (highest value)	
			Maxim value	At time biopsy		ALT	GGT
Epidermal damage score	r=0.03	r=0.17	r=-0.04	r=0.01	r=-0.06	r=0.22	r=-0.03
Lymphocyte score	**r=0.44***	r=0.12	**r=0.51**	r=-0.01	**r=0.65****	**r=0.51***	r=0.38
Eosinophil score	**r=0.44***	r=0.36	r=-0.02	r=-0.04	r=-0.18	r=-0.08	r=-0.01
Neutrophil score	r=0.3	r=0.29	r=0.3	r=0.21	r=-0.16	r=-0.23	r=-0.3

*p≤0.05; **p≤0.01

There were no significant differences in histopathological parameters between cases induced by allopurinol and the anticonvulsants.

The mean epidermal damage score was higher in biopsies from the 8 cases induced by allopurinol (2.81 ± 0.72 *vs.* 2.00 ± 0.27; *p>0.1*) whereas the mean Ly score was higher in the 5 cases induced by anticonvulsants (10.60 ± 2.18 *vs.* 9.37 ± 0.78, *p>0.1*), but the differences were not statistically significant.

Two cases of allopurinol-induced DRESS had no eosinophils in skin biopsies (pts 9 and 11), but, in general, allopurinol-induced cases had the highest eosinophil scores. Nevertheless, the mean Eos score was identical to the anticonvulsants (3.0 ± 1.03 and 3.13 ± 0.83, respectively for allopurinol and anticonvulsants; *p>0.5*). There was also no significant difference for the neutrophil score between the two groups of drugs (0.87 ± 0.43 *vs* 1.10 ± 0.37, respectively, for allopurinol and the anticonvulsants; *p>0.5*).

DNA for HHV-6 was evaluated in 6 patients with positive results in 3. There was no statistically significant difference between HHV-6 positive and negative cases, namely in which concerns the scores for epidermal damage (respectively, 3.33 ± 1.45 and 3.00 ± 0.87; $p>0.5$), the lymphocyte score (respectively, 12.00 ± 3.33 and 9.67 ± 2.09; $p>0.5$) and the eosinophil score (respectively, 5.00 ± 1.76 and 1.17 ± 0.60; $p=0.108$). Nevertheless, the neutrophil score was significantly lower in HHV-6+ patients (0.17 ± 0.17 *vs.* 2.17 ± 0.60; $p<0.05$).

Two HHV-6+ patients were the only ones with a significant periadnexal infiltrate of lymphocytes and eosinophils. The other case with HHV-6 reactivation presented with a pseudolymphomatous pattern.

No histological parameter was significantly different between the 3 patients with erythroderma and the others with erythemato-macular and papular eruption, namely in which concerns the degree of exocytosis of inflammatory cells, epidermal damage score and the scores for lymphocyte, eosinophil or neutrophil infiltration.

D.5. Discussion

D.5.1. Clinical characteristics of patients

This group of 15 patients reflects a relatively homogeneous group of DRESS cases, mainly definite DRESS cases. In two of the 5 cases of possible DRESS, a full investigation to exclude other causes was missing and, therefore, the DRESS score could not be increased to a definitive DRESS. Allopurinol and anticonvulsants, the main culprit drugs in the present study, are also those usually responsible for this pattern of CADR worldwide. Seven patients were genotyped and carry the HLA that confers a high risk for DRESS induced by allopurinol or carbamazepine, respectively, HLA-B*58:01 in 6/8 and HLA-

A*31:01 in 1/3 patients (McCormack et al. 2011),(Chiu et al. 2012),(Yun et al. 2014).

As previously reported in the literature, in 2 of our cases, amoxicillin administered during the initial symptoms of DRESS, may have contributed to the final reaction. This antibiotic can be an important concomitant stimulus in allopurinol and carbamazepine induced drug reactions (Boston Collaborative Drug Surveillance Program 1972),(Ben Fredj et al. 2010) or, as previously mentioned, amoxicillin may have triggered viral reactivation, therefore inducing an anti-viral T cell response and contributing to the DRESS manifestations (Mardivirin et al. 2010).

These DRESS patients share, with most published cases, the long latency period between drug intake and the onset of the adverse reaction, the long lasting reaction with fever, exanthema, enlarged lymph nodes, eosinophilia and liver damage that regressed on corticosteroids and drug withdrawal. Although DRESS is a potentially life-threatening CADR, we observed no fatal outcome related with DRESS (Chiou et al. 2008),(Chen et al. 2010),(Natkunarajah et al. 2011),(Walsh and Creamer 2011),(Husain et al. 2013).

An exuberant case of herpes zoster, observed in one of our patients after recovery (pt 4), has also been documented after DRESS in other series (Shiohara et al. 2010),(Kano et al. 2012)

D.5.2. Histopathologic characteristics of the exanthema

In the present work, the histological changes observed in cutaneous lesions of DRESS were rather heterogeneous, thus far, with no specific or characteristic histopathologic pattern, as reported in most studies that refer briefly to histopathology of DRESS. A perivascular lymphocytic infiltrate and occasional eosinophils in the upper dermis, dermal edema, extravasated erythrocytes, lymphocyte exocytosis, basal cell vacuolization and dyskeratosis are observed in varying degrees and distinct combinations, in our patients and in other series

(Chiou et al. 2008),(Chen et al. 2010),(Natkunarajah et al. 2011),(Walsh et al. 2013).

The histological patterns are generally described as an erythema multiforme-like type when the epidermal damage is greater (Chiou et al. 2008),(Chen et al. 2010), as a spongiotic dermatitis type, as a dermal inflammatory vascular reaction with vascular aggression manifested by edema and red blood cell extravasation without vasculitis (Walsh et al. 2013), as a lichenoid-type dermatitis (Hashizume and Takigawa 2005),(Chiou et al. 2008) or as a pseudolymphoma-like reaction (Chiou et al. 2008),(Husain et al. 2013), as generally observed in our cases.

The predominance of one pattern over the other, reported in the different studies, is not apparently related with the culprit drug, evidence of viral reactivation or the day of evolution of the DRESS. In most previously published cases, there is no report of the macroscopic morphology and the age of the biopsied lesion, and this may be important, as the exanthema is not a static process.

In DRESS, exanthema usually progresses from head to toes for a few days, with changing macroscopic features of the lesions. They vary from macular to papular and urticarial, may associate purpura, vesicles or bullae, and finally progress to desquamation. The skin biopsy will register only a moment in this dynamic evolution. Conscious of these limitations, we studied the variation of the histologic characteristics of DRESS with the day of evolution when the skin biopsy was performed, which showed a tendency to an increasing intensity of the lymphocyte and eosinophil infiltration, but no effect on the intensity of epidermal aggression. We found no other reports of the modification of the histopathologic parameters with the evolution of the exanthema, but in such a prolonged reaction we may suspect that, while the reaction is still increasing, more inflammatory cells will infiltrate the skin. They may, nevertheless, not be committed to induce necrosis of keratinocytes combined with the drug, as epidermal damage was not increasing in biopsies from more long-standing lesions. This discrepancy between dermal lymphocyte infiltration and

epidermal damage at a later stage may, eventually, be explained by a late virus reactivation within dermal monocytoid cells and, consequently, a more intense anti-viral T cell response (Hashizume et al. 2013).

Most of our cases (93.3%) showed significant epidermal damage, with isolated or, occasionally, confluent necrotic keratinocytes and/or vacuolization of the basal layer. Nevertheless, the intensity of these findings was consistent with an erythema multiforme-like pattern only in 3 cases (20%). This pattern was similarly observed in 9 out of 27 patients (33.3%) in the series of Walsh *et al.* (2013), but it was the main pattern described in the 60 cases described by Chen *et al.* (2010).

The spongiotic pattern with lymphocyte exocytosis similar to the one observed mainly by Walsh *et al.* (2013), occurred most particularly in 5 of our patients. A lichenoid pattern with epidermal aggression of the basal layer, observed most prominently in 2 cases, was also described in other series (Chiou et al. 2008),(Natkunarajah et al. 2011).

We found only one reference to a case with significant periadnexal or perifollicular infiltrate (Natkunarajah et al. 2011), a pattern that we observed in 2 of the 3 cases with a documented HHV-6 reactivation.

The inflammatory infiltrate in DRESS is usually considered denser than in other drug reactions and described as pseudo-lymphoma, particularly in the initial descriptions of the anticonvulsant hypersensitivity syndrome (Shiohara et al. 2007),(Husain et al. 2013). A pattern resembling this pseudolymphomatous reaction occurred only in a biopsy from a patient with a relapse during the course of carbamazepine-induced DRESS (day16), with aggravation of the erythemato-papular eruption, liver cytolysis and HHV-6 viral reactivation. We observed a dense, band-like lymphocyte infiltration with atypical cells, mitotic figures and lymphocyte exocytosis (Figs. D.6 g,h and D.7 b-g). There were no epidermal vesicles or nests of lymphoid cells resembling the typical Pautrier microabcesses of mycosis fungoides, but this aspect was

observed in a positive patch test from abacavir in a patient with DRESS (Fig. E.7) (Gonçalo et al. 2011).

The significant positive correlation between the intensity of the inflammatory infiltrate and the delay in taking the skin biopsy, may, eventually, explain the high frequency of the pseudolymphomatous pattern reported in the initial descriptions of the anticonvulsant hypersensitivity syndrome (Husain et al. 2013). In the first cases, a delay in formulating the correct diagnosis and suspending the culprit drug might also be associated with late biopsy collections and, therefore, with this predominant pseudolymphomatous pattern.

D.5.3. Histopathology of DRESS and overlapping features with other exanthematic drug eruptions

In general, there was a wide variability in the histopathology of the exanthema in DRESS. It shared many aspects with the usual presentation of the maculopapular exanthema with no systemic symptoms (Seitz et al. 2013) and, also, with other patterns of CADR.

In a recent review of 60 patients with maculopapular exanthema, Naim and co-workers reported dermal and epidermal changes similar to ours, mainly with a spongiotic dermatitis associated with interface dermatitis and vacuolar degeneration of basal cells (Naim et al. 2011). A lymphocyte infiltrate in the perivascular and interstitial, middle and upper dermis, was observed in all their patients, as in our DRESS cases. Eosinophils and neutrophils were present in 60-70% of their cases and, similarly, in 67-80% of ours. The authors refer turgescent endothelial cells with neutrophils inside the blood vessels and no signs of vasculitis (Naim et al. 2011), also observed in our patients.

Concerning epidermal changes, exocytosis, mainly of lymphocytes, and spongiosis was reported, respectively, in 100% and 97% of their cases (Naim et al. 2011), which is very similar to our data in DRESS, with lymphocyte

exocytosis and spongiosis, respectively, in 87% and 80% of the histologic slides. Our cases seem to have more keratinocyte aggression, manifested both by vacuolization of the basal layer and, particularly, by necrotic keratinocytes. These aspects, observed in 80% of our DRESS cases, occurred only in 20% of the cases of maculopapular exanthema (Naim et al. 2011). Moreover, we observed some cases of confluent keratinocyte necrosis, which was not reported in the study of maculopapular exanthema, and is more typically reported in SJS and TEN.

Actually, in patient 4, we observed keratinocyte necrosis of almost the full thickness of the epidermis, as in TEN. It was a severe case of DRESS that, clinically, also shared some aspects with a TEN at some time of its evolution: the patient developed some flaccid bullae, mainly on the upper arms and thighs, but with no Nikolsky's sign or atypical targets, and also lip erosions, although without oral or ocular mucosal erosions (Fig. D.2).

Neutrophil exocytosis and formation of subcorneal pustules, reminiscent of the pattern described in AGEP (Sidoroff 2012), is not usually observed in DRESS. It was, nevertheless, described in a patient with DRESS induced by carbamazepine that, in the initial phase, simulated an AGEP induced by antibiotics, both clinically and on histopathology (Matsuda et al. 2013). We also observed subcorneal spongiform pustules in patient 1, who presented with an exfoliative erythroderma, with no clinically visible pustules.

The different histologic patterns observed in the exanthema as a manifestation of DRESS, sharing aspects with maculopapular exanthema, AGEP, lichenoid drug eruptions, erythema multiforme and the more severe CADR with epidermal necrolysis, SJS and TEN, may reflect the clinical variability of the exanthema and, also, a possible continuum within the spectrum of these immune mediated delayed CADR.

Although the clinical patterns of delayed CADR have been well characterized and standardized phenotypically (Pirmohamed et al. 2011), occasional cases with overlapping criteria between these well established patterns have been

observed in multicentre studies (Bouvresse et al. 2012). Moreover, in the clinical practice we regularly observe cases of maculopapular exanthema that are associated with a few systemic symptoms, like mild fever and slight liver cytolysis but with no criteria for DRESS. On the other hand, other cases of maculopapular exanthema progress either to a full-blown DRESS or to SJS/TEN. Still, some cases of AGEP have exuberant skin detachment, resembling TEN (Peermohamed and Haber 2011) and, in occasional cases, pustules, or even an eruption simulation AGEP, has been described in DRESS (Matsuda et al. 2013) and in TEN (Liu et al. 2013).

All these CADRs represent the final result of the tissue aggression orchestrated by drug specific $CD8^+$ T cells and, also, $CD4^+$ T cells, although with some difference in the participation of distinct sub-phenotypes of effector T cells and co-factors (Pichler 2007),(Pichler et al. 2011),(Gonçalo and Bruynzeel 2012). Therefore, we may assume that some overlap can occur between the more phenotypically defined patterns of CADR and, consequently, that some overlap within the histopathology patterns may also be observed.

D.5.4. Histopathology and underlying pathophysiologic mechanisms

Drug specific cytotoxic $CD8^+$ T cells that recognize the drug bound to HLA class I molecules are considered some of the most important effector cells in the exanthema, particularly in DRESS from abacavir, allopurinol and carbamazepine (Chessman et al. 2008),(McCormack et al. 2011),(Adam et al. 2011),(Bell et al. 2013). $CD8^+$ T cells and cytotoxic NK cells have been shown to infiltrate skin lesions in DRESS and, by analogy with SJS/TEN (Nassif et al. 2004a), it might be expected that they induce apoptosis or necrosis of the keratinocytes that eventually bind the drug. Epidermal damage, observed in a high proportion of our cases, may reflect such a cytotoxic effector mechanism,

but we did not characterize the phenotype of lymphocytes infiltrating the skin in our patients.

Nevertheless, we observed no significant correlation between the intensity of keratinocyte necrosis or vacuolization of the basal layer and the intensity of the dermal inflammatory infiltrate or the exocytosis of lymphocytes or other inflammatory cells. This discrepancy has also been described in SJS/TEN, where, despite the intense epidermal necrosis, the dermal inflammatory infiltrate can vary from an almost "empty dermis" up to a dense dermal lymphocyte infiltrate, therefore, with an irregular correlation between the density of the infiltrate and the severity of epidermal damage (Quinn et al. 2005),(Valeyrie-Allanore et al. 2013).

The discrepancy between the intensity of inflammatory cells and epidermal aggression was explained, in TEN, as $CD8^+$ and other cytotoxic cells often leave the dermis to the epidermis and are found mainly in the blister fluid (Correia et al. 1993),(Cleach et al. 2000), which is not plausible to occur in DRESS, as there are usually no bullae. Nevertheless, both in TEN and DRESS, cytotoxic cells can induce epidermal aggression through their mediators, without direct contact. Apart from the secretion of perforin and granzyme B, that have a short acting range, other soluble mediators like granulysin, TNF-α and related death molecules, and soluble Fas-Ligand may induce keratinocyte killing at some distance from the secreting cell, as observed in SJS/TEN and, very probably, also in DRESS (Nassif et al. 2004a),(Chung et al. 2008),(Abe et al. 2009),(Schlapbach et al. 2011b),(Araujo et al. 2011).

Elevated granulysin levels, observed in the skin and blood in DRESS, although not so significantly as in SJS/TEN, also suggest the participation of this very efficient cytotoxic mediator in DRESS (Saito et al. 2012). Nevertheless, $CD8^+$ cells from DRESS patients seem different in their cytotoxic properties from those in TEN, justifying the usual absence of massive epidermal necrosis in DRESS (Hashizume and Takigawa 2005).

In DRESS, apart from $CD8^+$ cells, $CD4^+$ T cells also produce high levels INF-γ, TNF-α, IL-6, IL-5 and IL-17E, which may also contribute to the epidermal

damage. These cytokines, particularly IFN-γ and IL-17, increase the expression of receptors that make keratinocytes more susceptible to death induced by other mediators (Pennino et al. 2010),(Hirahara et al. 2010),(Picard et al. 2010),(Saito et al. 2012),(Ogawa et al. 2013).

Moreover, if in DRESS some of the skin infiltrating $CD8^+$ T cells are directed against viral infected cells in the dermis, keratinocyte aggression may be discrete and the dermal infiltrate may be intense, as in our three cases with documented HHV-6 reactivation, namely in patient 9 who presented with a pseudolymphomatous dermal infiltrate and a slight epidermal damage. Like CMV, that was observed in mononuclear dermal cells in DRESS (Hashizume and Takigawa 2005), HHV-6 also localizes in dermal cells, as recently shown by Hashizume and co-workers (Hashizume et al. 2013). Apparently, HHV-6 is latent in circulating myelomonocytic precursors that infiltrate the dermis and, upon arrival at the skin, the virus infects dermal T cells, particularly dermal $CD4^+$ T cells (Meyding-Lamadé and Strank 2012),(Hashizume et al. 2013), that may be the target for a potent anti-viral T cell response, justifying a dense lymphocytic dermal infiltrate.

On the other hand, in cases where the reactivated virus in DRESS localizes also in epidermal cells, as shown in cases for herpes simplex virus associated erythema multiforme (Burnett et al. 2008), a more intense epidermal necrosis might be expected.

Skin hybridization techniques to localize more precisely HHV-6 genome in skin cells, or immunohistochemistry to localize HHV-6 antigens, in correlation with the distribution of inflammatory cells, would be very important to understand better the relation between the anti-viral T cell response and skin lesions in DRESS and, eventually, the relation with a particular histologic pattern.

D.5.5. Histopathology in correlation with severity markers

We observed a statistically significant correlation, though moderate, between the density of the lymphocyte infiltrate in skin biopsies and the intensity of liver cytolysis. Liver toxicity is one of the most frequent and severe, occasionally life-threatening, manifestations of internal organ involvement in DRESS and it may occur only late in the course of this CADR. Therefore, scoring for lymphocyte infiltrate in a skin biopsy, in DRESS, can be an important prognostic marker. Nevertheless, as we found no other reports to reinforce this correlation, larger studies will be needed to replicate this finding. Otherwise, this may be a fortuitous isolated observation, reminding us of the possible relation between the intensity of the skin inflammatory infiltrate and the prognosis of SJS/TEN, that could not be further confirmed (Quinn et al. 2005),(Faye et al. 2005),(Valeyrie-Allanore et al. 2013).

In another study, Walsh *et al.* reported a good correlation between histopathology parameters and liver disease, but the correlation was between epidermal necrosis in skin biopsies and the intensity of liver cytolysis (Walsh et al. 2013), which may depend on a common type of aggression mediated by cytotoxic T cells. In our study, this correlation between epidermal damage and liver cytolysis was rather weak, although our patient 4, with the most intense epidermal damage, also had the most intense liver cytolysis.

Discrepancy between skin and systemic manifestations may occur because circulating drug specific T cells in DRESS are mostly $CCR4^+$ and CLA^+ skin homing T cells (Naisbitt 2004),(Tapia et al. 2007),(Ogawa et al. 2013). Therefore, drug reactive T cells may migrate preferentially to the skin and not to other organs, unless they are previously inflamed and express high amounts of adhesion molecules in their endothelial cells. In DRESS, as well as in other CADR, the drug may be presented to the immune system, either in the skin draining lymph nodes or in other lymphoid tissues and, consequently, sensitized drug specific T cells will have different homing capacities. Additionally, drug specific T cells are usually polyclonal and, apart from

different homing capacities, they may have similar or different effector phenotypes, favouring similar or distinct types of aggression in the different target organs to where they migrate.

Therefore, correlation between skin cytotoxicity and liver cytotoxicity, or other skin lesion and internal organ damage, may depend on the migratory capacity of effector T cells to the target organs and on their respective phenotype, which may be highly variable. Nevertheless, the positive correlation suggested in the study of Walsh *et al.* (2013) and a weak correlation (r=0.22) observed in our study, has certainly to be explored further in a larger cohort of patients. In case any of the reported correlation is further reproduced and strengthened, either between the intensity of the epidermal damage or the lymphocyte infiltrate and liver toxicity, an early skin biopsy may be proposed as a good and widely available prognostic tool to be used in DRESS.

Eosinophilia is one of the hallmarks of DRESS. Lymphocytes that produce high amounts of IL-5, CCL11/eotaxin and IL-17E are considered responsible for eosinophil recruitment into affected organs, where activated eosinophils are supposed to mediate tissue damage (Poszeczynska-Guigné et al. 2005),(Choquet-Kastylevsky et al. 1998). Actually, in 12 out of 15 (80%) of our cases, eosinophils were observed in skin biopsies, mostly in the perivascular and interstitial dermis, but the intensity of eosinophil infiltration was highly variable (scores 0 to 7). Eosinophils were mostly absent from the epidermis and dermal epidermal junction, and there was no correlation between their presence in the cutaneous infiltrate and the degree of basal cell vacuolization or keratinocyte necrosis. As eosinophils are considered one of the effector cells in skin manifestations of DRESS (Poszeczynska-Guigné et al. 2005),(Choquet-Kastylevsky et al. 1998), we might suspect that, after degranulation and loss of the eosinophilic cationic protein, they are no longer detected by the haematoxylin-eosin staining. This might also explain, in our cases and others, the frequent lack of correlation between the number of circulating eosinophils

and the quantity of eosinophils detected in skin biopsies (Chen et al. 2010),(Walsh et al. 2013). Detection of eosinophil cationic protein in the skin biopsy, by immunohistochemistry, might be an additional tool to evaluate the real correlation between circulating and skin infiltrating eosinophils, and might also allow a better understanding of the function of eosinophils in this CADR.

Eosinophilia in DRESS seems to be related with circulating IL-5, produced by drug-specific T lymphocytes, and with CCL17/TARC, produced in high amounts by dermal dendritic $CD11c^+$ cells (Ogawa et al. 2013). The chemokine CCL17 is significantly increased in the blood during the acute stage of DRESS and its levels are in good correlation with the circulating eosinophil count. Moreover, CCL17 levels also correlate with the intensity of erythroderma, possibly because this chemokine also recruits $CCR4^+$ T cells into the skin (Ogawa et al. 2013). This double effect of CCL17 on eosinophils and skin homing T cells, may also explain why we observed a moderate, but statistically significant correlation, between the intensity of lymphocyte infiltration in skin biopsies (Ly score) and eosinophilia (r=0.51).

D.6. Conclusions

In this work we performed a detailed study of the histopathology of skin lesions in 15 cases of DRESS, mostly with a high score but with no fatal outcome. We found no specific pattern or particular characteristic that might be sufficient to perform, or even confirm, a diagnosis of DRESS.

We observed a high variability of histopathologic changes, which may depend, at least partially, on the evolution of the DRESS at the time of biopsy. The different histopathologic patterns do not seem to correlate, either with the clinical morphology of the skin lesions, or the clinical and laboratory parameters observed during the acute state.

There was a moderate positive correlation between the intensity of the lymphocyte infiltrate and both systemic eosinophilia and hepatic cytolysis, but only a weak positive correlation between the degree of epidermal damage and other parameters of DRESS severity (hepatic cytolysis). Therefore, for the moment, skin histology cannot be used as a good definitive prognostic marker in DRESS. Nevertheless, if larger studies with more cases and, particularly with more severe cases, confirm the correlation between the intensity of the lymphocyte infiltrate and hepatic cytolysis, observed by us, and the association between keratinocyte necrosis and hepatic cytolysis reported by Walsh and co-workers (Walsh et al. 2013), skin histopathology in DRESS can become an important tool for predicting its prognosis.

The observation of basal cell vacuolization and keratinocyte necrosis and a positive correlation between this manifestation of epidermal damage and the intensity of the lymphocyte infiltrate, suggest that inflammatory infiltrating cells may exert a cytotoxic aggression on epidermal cells combined with the culprit drug, although not so intense as in TEN.

We found no particular pattern or histopathologic aspect that could be correlated, either with the causative drug or the presence of viral reactivation, apart from the peculiar periadnexal infiltration in HHV-6+ patients. If classical histopathology could be complemented with other techniques to characterize the phenotype of infiltrating cells and precisely localize viral DNA or, eventually, the culprit drug, it would certainly be an important tool to understand better the mechanisms underlying cutaneous changes observed in DRESS.

The wide spectrum of histopathologic changes observed and the variable macroscopic morphologic features of the exanthema observed in our patients with DRESS, may support the existence of some overlapping features between DRESS and other patterns of immune mediated delayed CADR, namely maculopapular exanthema, SJS/TEN and AGEP, as previously suggested (Bouvresse et al. 2012) and that we are presently studying (Gouveia et al. 2013).

Although there is usually a very good definition of these classical patterns of nonimmediate CADR, overlapping syndromes are somehow expectable, as the same or similar drugs can induce all these reactions and drug reactive T cells, particularly $CD8^+$ T cells, are mostly involved in the different skin and systemic lesions of these CADR.

E.
CLINICAL STUDIES.
II - HISTOPATHOLOGY OF POSITIVE PATCH TESTS IN CUTANEOUS ADVERSE DRUG REACTIONS

E. CLINICAL STUDIES. II - HISTOPATHOLOGY OF POSITIVE PATCH TESTS IN CUTANEOUS ADVERSE DRUG REACTIONS[2]

E.1. Introduction

Patch testing is a diagnostic technique used since the end of the 19th century, mainly in the study of allergic contact dermatitis (ACD). It is routinely performed for defining the aetiological diagnosis of ACD and it has been used, in experimental settings, to study the pathophysiology of ACD, both in man and animals.

For the last decades, patch testing has also been used more regularly in the study of delayed CADRs. Actually, patch testing was first used in the study of a drug reaction, a generalized acute eczematous dermatitis after a mercury injection for treating syphilis. Originally called "Funktionelle Hautprufung", this technique was developed by Joseph Jadasshon at the University of Breslau and first presented, in 1895, during the Congress of the Austrian Society of

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[2] The results of this chapter have been partially published as isolated case reports or in proceedings of Dermatological meetings as:

Histopathology of positive skin tests in different drug eruptions. Margarida Gonçalo, D Serra, AR Cabral, JP Reis, O Tellechea. J Invest Dermatol 2011: 131: S33, and Contact Dermatitis 2012: 66: S2: 21

Positive patch test in toxic epidermal necrolysis with clinical and histopathological aspect typical of TEN. Margarida Gonçalo, F Santiago, MJ Julião, O Tellechea. Contact Dermatitis 2010: 63: S1: 22-23

Acute generalized exanthematous pustulosis associated with acyclovir, confirmed by patch testing. David Serra, L Ramos, A Brinca, M Gonçalo. Dermatitis 2012: 23: 99-100

Pustular psoriasis and drug-induced pustulosis. David Serra, M Gonçalo, A Mariano, A Figueiredo. G Ital Dermatol Venereol 2011: 146 : 155-8.

Dermatology. Josef Jadassohn is considered the "father" of patch testing and the official birth of this method is reported to 1896, when the congress proceedings were published (Lachapelle 2011).

In the 70's, after Felix and Comaich published an article in the Lancet on the value of patch testing in drug eruptions, several isolated cases or small studies were published reporting the capacity of patch testing to confirm the responsible drug in CADR. Initial publications dealt mainly with carbamazepine (Houwerzijl et al. 1977),(Camarasa 1985),(Silva et al. 1986) and penicillin allergy, a drug largely studied by Derk Bruynzeel and his group (van Ketel 1975),(Bruynzeel et al. 1985),(Bruynzeel et al. 1985).

Larger published studies on this subject appeared in the 90's and beginning of 2000 (Osawa et al. 1990),(Quirino et al. 1996),(Gonçalo et al. 1996),(Barbaud 2002),(Lammintausta and KorteKangas-Savolainen 2005b) and motivated the publication of the ESCD (European Society of Contact Dermatitis) guidelines for performing skin tests in drug eruptions, where we were one of the leading authors (Barbaud et al. 2001a).

Thereafter, with the availability of many standardized commercial test preparations with systemic drugs, patch testing has been used with increasing frequency in the study of drug eruptions.

Patch testing has revealed to be a safe method, with very exceptional cases of immediate reactions or reactivation of the CADR. It is highly specific, when using commercial available allergens, but not exempt of false positive reactions, particularly when testing with the powders of the drugs used by the patients (Gonçalo and Bruynzeel 2011),(Barbaud et al. 2013a).

Nevertheless, its sensitivity is rather low (<40%), either due to the incapacity to form the culprit drug metabolite in the skin or due to absence of concomitant "danger signals" present during the CADR. Eventually, an insufficient epicutaneous penetration from the patch test (incorrect vehicle or drug concentration) can also be responsible for false negative patch tests, although intracutaneous tests with delayed readings do not significantly increase sensitivity of skin tests, particularly with the penicillins (Romano et al. 2013).

The sensitivity of the patch test depends much on the culprit drug. Patch tests are usually negative with allopurinol and its active metabolite, oxypurinol (Vieira et al. 2004a),(Santiago et al. 2010), whereas positive patch tests with carbamazepine occur in more than 60-70% of the CADR (Santiago et al. 2010). Patch tests are also frequently positive in drug eruptions from aminopenicillins (Barbaud 2007),(Hjortlund et al. 2013),(Romano et al. 2013), pristinamycin (Barbaud et al. 2004), clindamycin (Pereira et al. 2011), fluorquinolones and tetrazepam (Camarasa and Serra-Baldrich 1990),(Pirker et al. 2002),(Barbaud 2009),(Barbaud et al. 2013a).

Sensitivity of the patch test also depends on the pattern of the CADR, with positive reactions occurring mostly in maculopapular exanthema, DRESS and fixed drug eruption (Santiago et al. 2010),(Andrade et al. 2011). Patch tests are seldom in positive in SJS/TEN (Wolkenstein et al. 1996),(Barbaud 2002).

Moreover, correlation of positive patch tests results with pharmacogenetic studies in CDAR from abacavir was essential for definitively recognizing the significant association between abacavir hypersensitivity and HLA-B*57:01 (Phillips et al. 2002),(Shear et al. 2008),(Phillips and Mallal 2009a). In the initial studies, this relation was not so clear, as many cases of exanthema in the AIDS population, highly prone to skin reactions, were incorrectly attributed to this anti-retroviral drug. Positive patch tests confirming the drug involvement reinforced the very strong association with HLA-B*57:01, allowed the definition of the real risks and supported very effective preventive measures that almost abolished hypersensitivity syndrome on abacavir exposure (Phillips et al. 2005),(Hughes et al. 2008),(Phillips et al. 2011), (Hashizume 2012).

As in ACD, apart from confirming the etiologic agent, patch testing has been used also to study pathophysiologic mechanisms involved in delayed CADR (Barbaud 2002),(Barbaud 2009),(Phillips and Mallal 2009a). Skin infiltrating cells in patch tests have been characterized at several time points of its evolution, drug specific T cells have been isolated from the patch tests and T cell lines and clones were established to study *in vitro* reactivity with the

responsible drugs (Posadas and Pichler 2007),(Yawalkar et al. 2000). Most T cells isolated from patch tests share specificity and phenotype markers with drug reactive T-cell clones isolated from the blood or from the skin, during the acute eruption (Yawalkar et al. 2000).

The objective of the present work was to characterize positive patch tests to drugs in different patterns of delayed CADR and caused by different drugs, both on their morphology and, particularly, on histopathology. It was our intention to evaluate how patch tests can reproduce, macro- and microscopically, the morphologic features of the different patterns of CADR. This might reinforce their potential use to study pathophysiologic mechanisms involved in the effector phase of immune mediated delayed CADRs.

E.2. Methods

E.2.1. Inclusion criteria and patient selection

For the last two decades, in the Department of Dermatology of the University Hospital, patients with delayed CADR are regularly patch tested with the objective of obtaining a possible confirmation of the culprit drug and detecting hypersensitivity to other related chemicals. With this data, and the report from the hospitalization or the out-patient data file, a medical report is given to the patient and his family physician, which includes the pattern and severity of drug eruption developed, the most probable or confirmed responsible chemical, the drug (or drugs) the patient has to avoid definitively, or preferentially, and alternative recommended safer drugs. Occasionally, in the case of negative patch test results and non severe CADR, namely in fixed drug eruption or maculopapular exanthema, intracutaneous tests or a controlled drug rechallenge with a low dose is performed in the hospital setting, under medical supervision.

We used the following inclusion criteria for the present study:

- non-pregnant patients, older than 18 years;
- patients who suffered and recovered completely from a delayed CADR that was followed, during the acute phase, at the Department of Dermatology of the University Hospital;
- patients with a well characterized CADR, according to the regularly defined phenotypes (Pirmohamed et al. 2011), and with only one or 2 culprit drugs, identified according to the French Pharmacovigilance criteria (Bégaud et al. 1985);
- patients who were patch tested at the Department of Dermatology, according to the guidelines of patch testing in drug eruptions (Barbaud et al. 2001a), and had positive reactions to the culprit drug; and
- patients that gave their informed consent for performing skin biopsy at the patch test site.

E.2.2. Patch testing. Methods and materials

Patients were patch tested at least 6 weeks after the complete resolution of the drug eruption and no longer than 6 months thereafter, according to the ESCD guidelines of patch testing in drug eruptions (Barbaud et al. 2001a).

Patch tests were performed with the suspected drug and chemically related substances or drugs belonging to similar pharmacological groups. Most tests were performed with pure drug allergens from Chemotechnique Diagnostics, Vellinge Sweden or Bial-Aristégui, Spain, in standardized concentrations, usually 1-10% in petrolatum.

When not commercially available, the powder of the drug with >95% purity, kindly supplied by the pharmaceutical industry, was prepared in our

department at 1-20% concentration in petrolatum and, occasionally, in other vehicles.

When we had no access to the pure product, we used in house fresh preparations made with the powder in the vials for i.v. administration, with the powder inside the capsules or, in its absence, preparations were made with crushed tablets. The powder was diluted in petrolatum, water or, occasionally, in other vehicles, in order to have a concentration of the active drug at 10% in the final preparation. When it was not possible to attain this concentration, due to the low amount of the active drug in the commercial preparation, the whole powder was prepared at 30% in petrolatum.

Allopurinol and oxypurinol, that are usually negative when patch tested at 1-20% in petrolatum, water, alcohol or acetone, were pre-solubilized in DMSO (dimethyl sulfoxide) and then incorporated in petrolatum. In these patients, patch tests were performed also with DMSO alone in petrolatum to compare with the same vehicle containing the active drug.

The allergens were applied for 48h, on the back, using 8 mm aluminium Finn chambers® mounted on Scanpor® tape, Epitest Ld Oy or IQ Ultra® square polyethylene foam chambers attached to hypoallergenic tape, Chemotechnique Diagnostic, Vellinge, Sweden, and covered with Mefix® tape or analogue.

Readings were performed twice, between day 2 (D2) and D7 after patch test application, usually with a common reading on D3. Readings were performed and rated according to ICDRG (International Contact Dermatitis Research Group) guidelines and scoring system (- or negative, IR or irritant, '?' or doubtful, and positive 1+, 2+ or 3+). A particular emphasis was put on the description of morphological aspects of the skin reaction, other than the erythema, infiltration, papules and vesicles, regularly observed in patch tests from contact allergens.

E.2.3. Skin biopsy collection and histopathology evaluation

After patient's informed consent, a 4 mm skin punch biopsy was performed at D3 or D4 on positive patch tests, after a local anaesthesia with 2% lidocaine without epinephrine.

Skin fragments were fixed on formalin for 24 hours, processed for routine light microscopy and coloured with haematoxylin and eosin.

Histology slides were randomly and independently analysed by two experienced Dermatopathologists with no knowledge of the clinical pattern of CADR or the morphology of the patch tests. Several morphological aspects were evaluated on skin histopathology, including the general reaction pattern and more specific details.

Within the epidermis, attention was taken to the characteristics of the horny layer, intensity of spongiosis, exocytosis, vesicles, pustules, vacuolar changes in the basal epidermal layer, keratinocyte necrosis and the main inflammatory cells in exocytosis in the epidermis. In the dermis, the evaluation concerned mainly the lymphocyte, eosinophil or neutrophil infiltrate and its main distribution (upper or mid dermis, perivascular, interstitial, dermal-epidermal junction or periadnexal), the dermal edema, vasodilation, intravascular cells and extravasated erythrocytes.

Similarly to previously reported, the two Dermatopathologists independently rated each of these parameters between 'zero' (as absent) and 6 (as very intense) but, in this study, the sum of their individual scores was calculated to grade the intensity of the parameter studied (maximum score 12). Different histopathologic aspects were correlated with the clinical pattern of the CADR, the macroscopic characteristics of the patch test and, also, with the drug causing the positive patch test, considering particularly the groups of anticonvulsants and antibiotics.

Additionally, in order to characterize the inflammatory infiltrate, paraffinated sections of skin biopsies from 2 cases were processed for

immunohistochemistry in the Pathology Department, using antibodies to T cell markers (CD3, CD8, CD56 and granzyme B).

E.3. Results I – Characteristics of the patients and the drug eruptions studied

Between 2008 and 2013, we studied 18 patients, 7 males and 11 females, aged 43-86 years (mean 60.3 ± 12.5 years) with different clinical patterns of delayed drug eruptions. The culprit drug(s) were considered as highly probable (C3S3I4) according to the French Pharmacovigilance criteria, as previously referred, and confirmed by patch testing.

CADR were caused by antibiotics in 9 patients, other antimicrobials in 2, anticonvulsants in 4, diltiazem in 2 and allopurinol in the remaining 2. All CADR developed more than 24 h after drug intake, and some only 40 days thereafter (mean 11.3 ± 11.0 days) (Table E.1).

In 5 patients, a diagnosis of AGEP was performed according to the RegiSCAR criteria (Halevy 2009). Patient 1, suffering from psoriasis vulgaris for many years, developed 3 accesses of generalized pustular rashes, with accentuation in large body folds, always shortly after using fluorquinolones to treat urinary tract infections. They were retrospectively diagnosed as AGEP, also based on patch test results. Apart from this case, induced by ciprofloxacin and other fluorquinolones, the other cases were caused by clindamycin, acyclovir (1 case each) and diltiazem (2 cases). AGEP developed within 1-7 days (mean 4.2 ± 2.8 days) after initiating therapy.

Maculopapular exanthema with no significant systemic symptoms occurred in 6 patients and was induced by antibiotics (amoxicillin - 3 cases, cefotaxime, cefoxitin and ciprofloxacin -1 case each). Skin reaction developed after a

latency period of 2 days up to 7 days (mean 4.0 ± 2.0 days).

The 5 cases of cutaneous exanthema that fulfilled RegiSCAR criteria for DRESS (Kardaun et al. 2007) were induced by carbamazepine (2), phenytoin, allopurinol and abacavir (1 each). Reaction developed 10 to 40 days after initiating therapy (mean 24.4 ± 10.9 days) and the exanthema was associated with hepatic cytolysis, eosinophilia, lymphadenopathy and aggravation of renal function or digestive symptoms (Table E.1).

One patient developed SJS, 21 days after the introduction of allopurinol at the highest dose (300mg/day) for asymptomatic hyperuricemia.

Patient 18 suffered TEN with detachment of more than 50% of the total body surface area. Carbamazepine used for the treatment of seizures from meningioma was introduced just after urgent brain surgery, 15 days before the initiation of the cutaneous reaction (Table E.1).

Table E.1 – Characteristics of the patients, the culprit drug, reason for its prescription, interval to the onset of the CADR and the pattern of the CADR that motivated patch testing

pt.	age	sex	culprit drug	reason for prescription	interval (days)	clinical pattern	particular aspects
1	65	F	ciprofloxacin	recurring UTI	1	AGEP	psoriasis; 2 previous pustular eruptions with fluorquinolones
2	53	M	acyclovir	herpetic retinitis	4	AGEP	
3	48	F	clindamycin	genital infection	7	AGEP	also used amoxicillin
4	49	M	diltiazem	arterial hypertension	2	AGEP	
5	57	M	diltiazem	arrhythmia	7	AGEP	
6	64	M	ceftriaxone	surgery-single dose prophylaxis	3	MPE	previous CADR after ceftrx. for polytrauma
7	86	F	amoxicillin		7	MPE	targets; elevated liver enzymes
8	73	F	ciprofloxacin	genital surgery prophylaxis	2	MPE	
9	43	M	cefazolin	erysipela	6	MPE	concomitant use of clindamycin for 5 days
10	53	F	amoxicillin	dental implant	3	MPE	facial edema, vesicles
11	60	F	amoxicillin	ORL complaints	3	MPE	
12	51	M	carbamazepine	epilepsy	20	DRESS (definite)*	MPE with atypical targets
13	48	M	abacavir (HAART)	AIDS	10	DRESS (definite)*	HLA-B*57.01+
14	69	F	phenytoin	sub-arachnoid haemorrhage	24	DRESS (probable)*	
15	70	F	carbamazepine + amoxicillin	cerebral haemorrhage	28	DRESS (definite)*	HLA-A*31.01+ CADR to phenytoin amoxicillin at initial DRESS symptoms
16	78	F	allopurinol	hyperuricemia	40	DRESS (definite)*	
17	73	F	allopurinol	hyperuricemia	21	SJS	liver cytolysis
18	46	F	carbamazepine	meningioma-post surgery	15	TEN	SCORTEN 2 at admission; ±50% BSA

UTI – urinary tract infections; ORL – oto-rhino-larynx; HAART – highly active anti-retroviral therapy; *according to RegiSCAR criteria these DRESS cases could be defined as probable or definite, respectively with 5 or >6 criteria (Kardaun et al. 2007); + HLA conferring a risk factor for severe CADR from abacavir and carbamazepine (Phillips and Mallal 2009a),(McCormack et al. 2011); SCORTEN – prognostic score for TEN (Quinn et al. 2005)

E.4. Results II – Macroscopic morphology of positive patch tests

We observed positive patch test reactions with the culprit drug and, sometimes, also with related substances in the 18 patients (Table E.2; Fig. E.1). Cross-reactions or concomitant positive reactions were observed among the betalactams and among the fluorquinolones. All 5 patients reacted both to ampicillin and amoxicillin, and one of them also to dicloxacillin and ceftriaxone. Among cephalosporin positive patients, one reacted both to cefotaxime and ceftriaxone and the other to cefoxitine and cefazolin. In 2 cases, positive reactions were observed both to carbamazepine and to amoxicillin and ampicillin.

In 14 cases, positive tests showed erythema and infiltration covering the whole patch test area, therefore, a definite positive reaction according to the ICDRG criteria for the diagnosis of contact allergy. In 5 cases the reaction extended beyond the patch test application area.

In patient 5, with AGEP, erythema did not cover the whole patch test area but there were scattered pustules in the area of application of diltiazem. In patient 8, erythema and papules were almost exclusively limited to the contact with edge of the test chamber (edge effect), but similar reactions occurred both with ciprofloxacin and norfloxacin, reinforcing the relevance of the reaction. An accentuation of the reaction at the edge of the positive patch tests also occurred in patient 2, in the patch tests performed with commercial creams containing acyclovir. In patient 14, erythema was very faint but infiltration and papules occurred in both tests with phenytoin at 5 and at 10% in petrolatum.

In 10 patients positive patch tests presented with papules and vesicles. In 5 patients with AGEP, pustules could be clearly identified on the patch tests, particularly at the D3 reading.

Patient 18, with a carbamazepine-induced TEN, tested with 3 different concentrations of carbamazepine, had an intense vesicular reaction with

confluence of the 3 different test areas and a central area of skin detachment and the typical Nikolsky's sign of TEN.

In patients 16 and 17, as we used DMSO for diluting allopurinol and oxypurinol, we observed irritant aspects in the reaction (shampoo effect), but the reaction with allopurinol and oxypurinol was significantly more intense than the control only with DMSO in petrolatum (Fig. E.9). Among the five controls tested with similar preparations, two showed a very faint irritant reaction, with a shampoo-like effect.

Skin biopsies on the reactive patch tests were performed at day 3 in all cases, except in patients 10 and 11 with maculopapular exanthema from amoxicillin, whose biopsy was collected at D4.

In patient 16, with DRESS from allopurinol, we performed 2 biopsies, one from a control patch test containing only DMSO in pet (73110a), and the other from a positive patch test where allopurinol had been pre-dissolved in DMSO (73110b).

Table E.2 – Patch test results (intensity and morphology) in the different CADR. Test material used and positive patch tests with other drugs

pt.	culprit drug	clinical pattern	PT number	test material	+ test	morphology	positive PT with other drugs
1	CIPF	AGEP	186/2008	CIPF 10% pet*	2+	papules (D2); flaccid pustules (D3)	norfloxacin 10% pet* lomefloxacin 10% pet**
2	ACY	AGEP	064/2011	Zovirax cream®	2+	pustules "edge effect"	other acyclovir containing topic drugs
3	CLID	AGEP	079/2013	CLID 10% pet*	2+	scattered papulo-pustules not covering all PT area	
4	DTZ	AGEP	103/2010	DTZ 10 % pet*	2+	infiltration with discrete pustules	
5	DTZ	AGEP	083/2011	DTZ 10 % pet*	1+	discrete erythema with pustule	
6	ceftriaxone	MPE	155/2011	cefotaxime 10%*	2+	vesicles exceeding PT area	ceftriaxone 10% pet***
7	AMX	MPE	191/2011	AMX 10% pet*	2+	papules and discrete vesicles	ampicillin 10% pet*
8	CIPF	MPE	088/2012	CIPF 10% pet*	1+	papules "edge effect"	norfloxacin 10% pet*
9	cefazolin	MPE	095/2012	cefoxitin 10% pet**	2+	papules discrete vesicles	cefazolin 10% pet*
10	AMX	MPE	008/2013	AMX 10% pet*	2+	vesicles exceeding PT area	ampicillin 10% pet*
11	AMX	MPE	090/2013	AMX 10% pet*	2+	vesicles exceeding PT area	ampicillin 10% pet* dicloxacillin 10% pet* ceftriaxone 10% pet*
12	CBZ	DRESS	251/2011	CBZ 1, 10% pet*	2+	papules discrete vesicles	ampicillin 10% pet* amoxicillin 10% pet*
13	abacavir (HAART)	DRESS	91/2011	Ziagen® 10%pet***	3+	confluent vesicles widely exceeding PT area	
14	PHY	DRESS	183/2012	PHY 10% pet*	1+	faint erythema papules	PHY 5% pet**
15	CBZ + AMX	DRESS	072/2012	CBZ 1, 10%pet*	2+	vesicles exceeding PT area	
16	ALP	DRESS	231/2011	ALP+ 10% pet	2+	vesicles	oxypurinol+ 10% pet
17	ALP	SJS	212/2012	ALP+ 10% pet	1+	infiltration with sharp limits	oxypurinol+ 10% pet
18	CBZ	TEN	149/2009	CBZ 1, 10%pet*	3+	exuberant reaction confluent vesicles	CBZ 20%pet**

PT – patch test; CIPF- cicprofloxacin, ACY-acyclovir, CLID- clindamycin, DTZ-diltiazem, AMX-amoxicillin, CBZ-carbamazepine, PHY-phenytoin, ALP-allopurinol; pet. - petolatum ;*- allergens from Chemotechnique Diagnostics; **- pure chemicals prepared in house; ***- freshly prepared powder of the pills; +allopurinol and oxypurinol dissolved in DMSO (dimethyl sulfoxide) and prepared in pet.

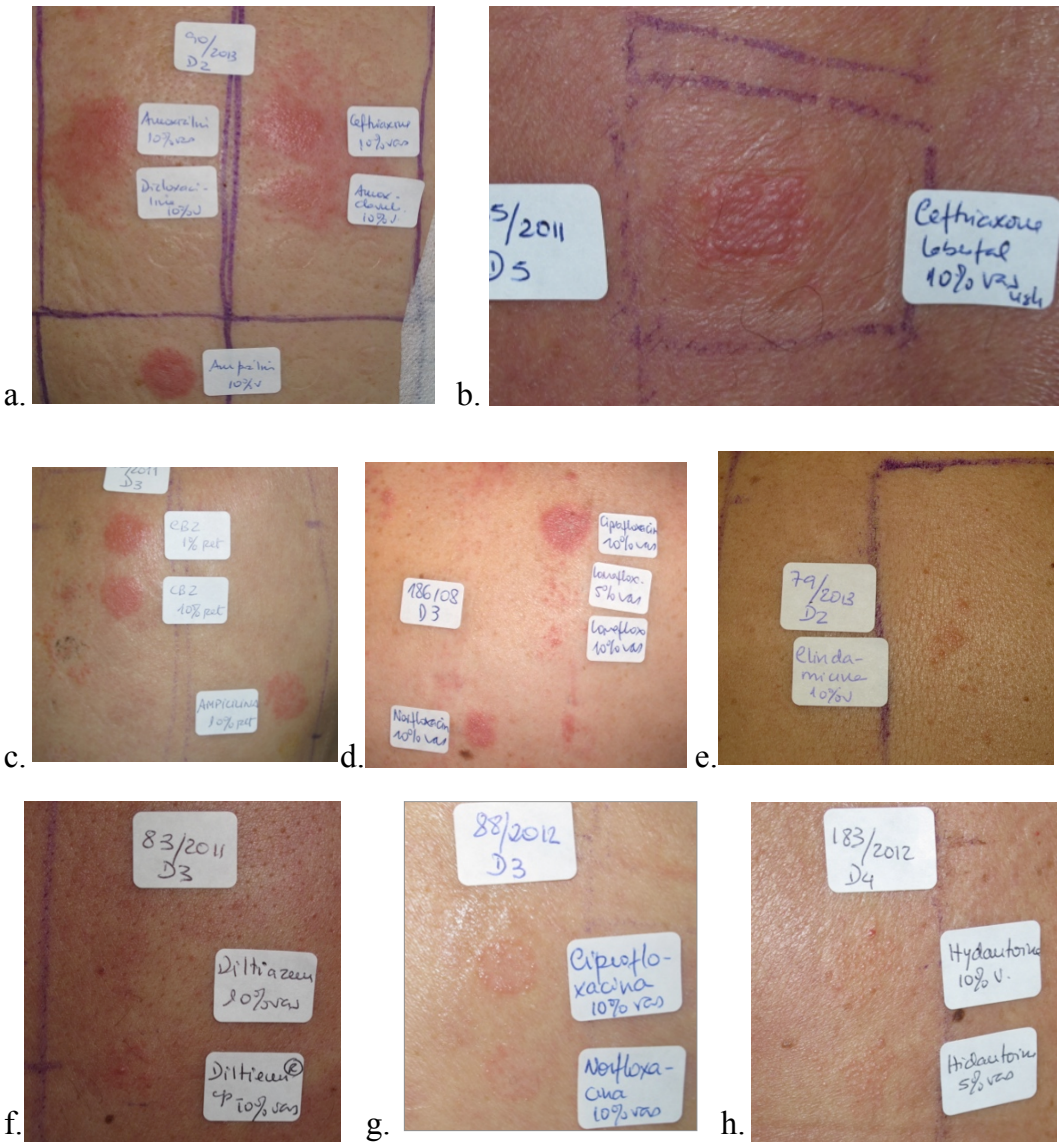


Figure E.1 – Macroscopic aspects of positive patch tests with drugs. Erythematopapular or vesicular reactions covering and exceeding the patch test application area in maculopapular exanthema from aminopenicillins and cephalosporins in pts 6 and 11 (a, b) and in DRESS from carbamazepine in pt 12, in this case with reactivity also to amoxicillin (c). Pustular reactions observed in AGEP from ciprofloxacin, norfloxacin and lomefloxacin in pt 1 (d), and less intense pustules with no underlying erythema in 2 cases of AGEP from clindamycin (e) and diltiazem (f). Less intense reactions from ciprofloxacin and norfloxacin with an "edge effect" in a MPE from ciprofloxacin in pt 8 (g) and a papulovesicular reaction with faint erythema to phenytoin in 2 concentrations in pt 14 (h).

E.5. Results III - Histopathology of positive patch tests

Histopathology of the patch tests showed epidermal spongiosis and exocytosis as well as a perivascular infiltrate in the upper dermis in all 18 patients and 19 biopsies taken, although with a highly variable intensity. Lymphocytes were the main inflammatory cell in the patch test biopsies, both in the dermis and in the epidermis. Neutrophils and/or eosinophils were occasionally observed. Particular aspects of the 18 drug patch test biopsies will be detailed below (Table E.3 and 4; Fig E.2-9).

E.5.1. Histopathology. Epidermal changes

The horny layer was orthokeratotic in 15 patients (83.3%) and parakeratotic in 3 patients (16.7%), although parakeratosis was only focal in 2 of them.

In the 3 biopsies from the tests using DMSO as the vehicle (cases 16a, 16b and 17), we observed a very peculiar aspect of the horny layer, even in the control patch test with no active drug. The orthokeratotic corneal layer presented with a prickly appearance with regularly distributed spikes at the deeper part, adjacent to the granulous layer, and a basket weave appearance at the outer horny layer (Fig. E.9). In these three biopsies, particularly in the areas of more inflammation, keratinocytes often showed, also, a vacuolar aspect with apparent shrinkage of the cytoplasm.

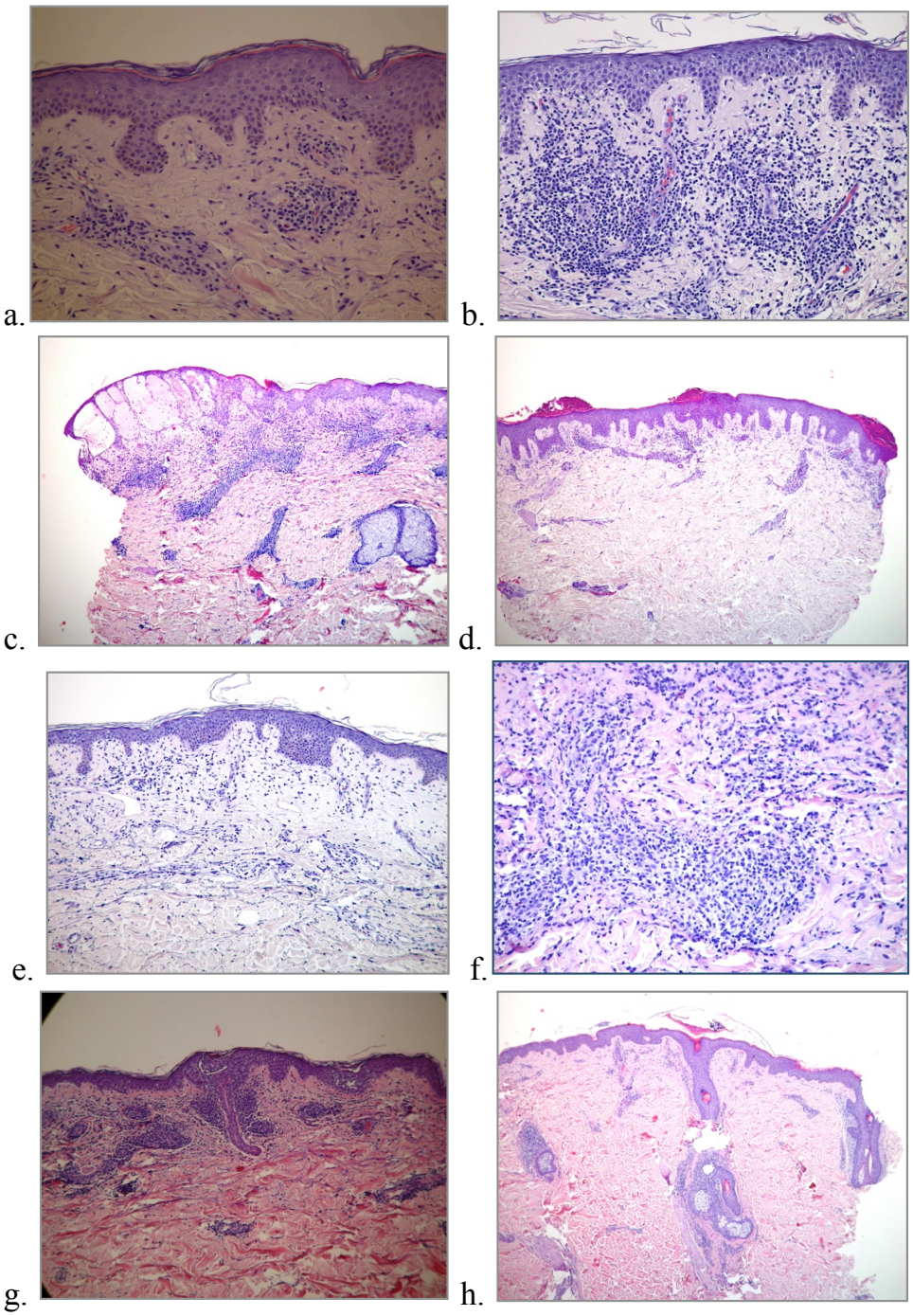


Fig. E.2 – **General histopathologic aspects of positive patch tests**. Orthokerathosis (a) and varying degrees of epidermal damage, namely exocytosis (b), vesicles (c) or pustules (d). The dermal infiltrate with a variable density, occurs mostly in the upper or mid-dermis, and is either diffuse (e) or mainly around blood vessels (c,d), eccrine (g) or follicular structures (h). Lymphocytes predominate, but eosinophils also occur (f) as well as neutrophils, particularly inside the capillaries and in exocytosis in pustular reactions (h)

Pustules were observed in 6 biopsies, either intracorneal and/or subcorneal, 4 of them from AGEP cases and two in maculopapular exanthema from amoxicillin and cefazolin.

In patient 1, with psoriasis and AGEP, cutaneous histology showed a subcorneal pustule during the acute CADR and a subcorneal and intraepidermal spongiform pustule in the patch test from ciprofloxacin (Fig. E.3). Acanthosis was reported in this patient, who suffered from psoriasis, and also in patient 2, with AGEP but no previous dermatological disease (Fig. E.3).

In another patch test from AGEP, with a very faint patch test reaction, no pustule was observed and, in the other case of AGEP induced by clindamycin, the pustule was surrounding the follicular infundibulum (Fig. E.3).

Coexistence of vesicles and pustules occurred in patient 9, with a positive PT to a cephalosporin. In this patient there was significant exocytosis, both of lymphocytes and neutrophils.

Vesicles were observed in all patch tests from DRESS and SJS/TEN and in 3 of the 6 cases from maculopapular exanthema, but were absent in AGEP. Vesicles were more intense in severe CADR (mean score 9.2 ± 3.2 and 5.5 ± 3.5, respectively in DRESS and SJS/TEN) than in maculopapular exanthema (mean score 1.7 ± 2.3) (Table E.5).

Vesicles occurred mainly and more intensely in patch tests from anticonvulsants, that also induced the more severe CADRs. No pustules were observed in the patch tests from anticonvulsants, whereas they occurred both in AGEP and in two cases of maculopapular exanthema induced by antibiotics (Table E.6).

Spongiosis varied from very mild in a case of AGEP (0.5) to very intense (score 10 and 12) in 2 cases of DRESS (Fig. E.6). Average score for spongiosis was generally more intense in the 5 cases of DRESS (7.4 ± 3.8) than in the 2 cases of SJS/TEN (5.5 ± 3.5) and in the 6 cases of maculopapular exanthema (5.0 ± 2.1)

or the 5 cases of AGEP (3.5 ± 0.6) (Table E.3 and 5). There was no difference in the score for spongiosis between antibiotics and anticonvulsants (Table E.6).

Exocytosis showed less variability in intensity, with mean scores of 4.2 ± 1.8, 5.3 ± 2.1, 6.0 ± 2.5 and 7 ± 1.4, respectively in AGEP, maculopapular exanthema, DRESS and SJS/TEN. Mean scores were also similar for antibiotics and anticonvulsants, respectively 5.2 and 6.2 (Tables E.5 and E.6).

Cells in exocytosis were predominantly neutrophils in 6 cases (33.3%), with exclusive neutrophil exocytosis in 2 cases of AGEP. Lymphocytes in exocytosis were observed in the other 16 cases (88.9%), exclusively in 11 cases (61.1%), and in association with eosinophils in 2 patch tests from DRESS.

In 3 patients, lymphocyte exocytosis occurred in small groups of cells forming nests, reminiscent of the Pautier's microabscesses described in mycosis fungoides (Fig. E.7). These microabscesses were very well formed in 2 DRESS cases (pts. 8 and 9) and less typical in a test from a maculopapular exanthema.

Clusters of Langerhans/histiocytic cells were observed around vesicles in 2 cases, one from a maculopapular exanthema (pt. 7) and another from DRESS (pt.14) (Fig. E.5 and E.6).

Vacuolization of basal keratinocytes occurred in 13 cases (72.2%), more intensely in SJS/TEN (mean score 6.6 ± 3.5) than in DRESS (mean score 2.8 ±2.9), or in the other CADRs.

Keratinocyte necrosis occurred in the 3+ positive patch test in the case of DRESS induced by abacavir (pt. 13) and, particularly, in the positive patch test from carbamazepine in TEN that, macroscopically, presented with confluent vesicles, skin detachment and Nikolsky's sign (pt. 18). In this patient, keratinocyte necrosis was very extensive and, in certain areas of the biopsy, involved the whole epidermal thickness. Occasional lymphocytes could be seen surrounding necrotic keratinocytes (Table E.3, 5) (Fig. E.8).

Table E.3 – Epidermal changes observed in skin biopsies of positive patch tests

pt	clinical pattern	histo-logy nr	horny layer	spon giosis	exocy tosis	main cells	vesi cles	pus tules	localization	necro sis	Vacuo lation
1	AGEP	64572	OtK	3	6	Neut	0	4*	intra-subcorneal	0	0
2	AGEP	71602	Pkt	3	6	Neut	0	10*	intra-subcorneal	0	2
3	AGEP	76790	OtK/ Pkt	4	2	Ly	0	3	follicullar infindibula	0	0
4	AGEP	69376	Pkt	4	4	Ly Neut	0	6	subcorneal	0	0
5	AGEP	71785	OtK	0.5	3	Ly	0	0		0	0
6	MPE	72406	OtK	8	4	Ly	4	0	acro-syringium	0	4
7	MPE	72805	OtK	4	7	Ly	1**	4	eccrine channel	0	8
8	MPE	74128	OtK	6	4	Ly	0	0		0	1+
9	MPE	74178	OtK/ Pkt	6	8	Ly Neut	5	4	intra-subcorneal	0	1
10	MPE	76089	OtK	4	6*	Ly	0	0		0	2+
11	MPE	76881	OtK	2	2.5	Ly	0	0		0	0.5+
12	DRESS	73380	OtK	4	8*	Ly Eos++	12	0		0	3
13	DRESS	71825	OtK	12	9*	Ly Neut Eos	12	0		5	6
14	DRESS	74806	OtK	3	3	Ly	2**	0		0	2+
15	DRESS	74027	OtK	10	6	Ly	12	0		0	0
16	DRESS	73110a	OtK***	8	2	Ly	6	0		0	1
		73110b	OtK***	8	4	Ly	8	0		0.5	3+
17	SJS	72982	OtK***	8	6	Ly	3	0		0	3+
18	TEN	66661	Pkt	3	8	Ly Neut	8	0		12	10
%			O-83% P-27%	100%	100%	L-88.9% N-33.3% E-11.1%	55.5%	33.3%		16.6%	61.1%

OkT: orthokeratosis; PkT: parakeratosis,
Ly: lymphocytes, Neut: neutrophils, Eos: eosinophils
73110 a – PT only with DMSO in pet (16a); 73110b - PT with allopurinol solubilized in DMSO (16b)
* lymphocyte exocytosis in groups of cells forming "microabcesses", similar to Pautrier's microabscesses;
**clusters of Langerhans cells within the vesicles;
*** horny layer with a regular spiculation;
+ only focal vacuolization;
++ very rare eosinophils.

E.5.2. Histopathology. Dermal changes

Dermal edema occurred in 15 patients (83.3%) and was particularly significant in the case of TEN.

Dermal inflammatory lymphocyte infiltrate occurred in all cases, mainly in the upper dermis, but it extended also to the reticular dermis in 8 cases (44.4%), and still deeper in one case.

The inflammatory infiltrate was mainly perivascular (18 - 100%), interstitial (15 - 83.3%) and at the dermal epidermal junction (14 - 77.7%) and, also, around cutaneous adnexa (9 - 50%), namely around the follicles (4 – 22.2%), eccrine glands (4 – 22.2%), both at the acrosyringia and the deeper lobules, and around nerves (1 – 5.5%). The inflammatory infiltrate was composed mainly of lymphocytes, with no atypical cells, and was associated with neutrophils in 3 cases (2 AGEP and 1 maculopapular exanthema) or eosinophils in 4, particularly in the interstitial dermis.

The inflammatory infiltrate, particularly in the interstitial dermis, was more intense in DRESS and SJS/TEN than in maculopapular exanthema or AGEP (Table E.5). If we consider the sum of the average score for the inflammatory in the different dermal localizations, it increased progressively from AGEP (7.0), to maculopapular exanthema (12.8), to DRESS (13.4) and to SJS/TEN (18.0).

There was no particular difference in the intensity of the dermal inflammation between the groups of patch tests from antibiotics or anticonvulsants (Table E.6).

There were no signs of vasculitis, although extravascular erythrocytes were observed in 8 cases (44.4%) and discrete leukocytoclasia was observed in patient 9, with a positive patch test from a cephalosporin.

Intravascular erythrocytes or neutrophils could be seen in all patients, in one case very well aligned along a blood vessel (Fig. E.2.b).

Table E.4 – Dermal changes observed in skin biopsies of positive patch tests

pt	clinical pattern	histo-logy nº	localization	edema	Dermal infiltration						extrav RBC
					peri vasc	inters titial	DEJ	periadnexal		main cells	
1	AGEP	64572	DS	4	3	3	2	0		Neut Ly	0
2	AGEP	71602	DS	4	6	5	0	0		Ly	0
3	AGEP	76790	DS/DR	0	2	0	0	7	folic	Ly	0
4	AGEP	69376	DS	3	6	4	0	0		Ly Neut	0
5	AGEP	71785	DS	2	4	0	0	0		Ly	0
6	MPE	72406	DS	6	9	5	3	2	acr-syr	Ly Eos	0
7	MPE	72805	DS++/DR	0	8	4	2	5	acr-syr	Ly	2
8	MPE	74128	DS	3	7	3	2	0		Ly	1
9	MPE	74178	DS++/DR	2	6	4	3	2	folic	Ly Neut	6
10	MPE	76089	DS	0	6	0	3	0		Ly	0
11	MPE	76881	DS++/DR	2	5	6	1	8	folic	Ly Eos	1
12	DRESS	73380	DS++/DR	1	7	2	3	0		Ly	10
13	DRESS	71825	DS/DR/DD	6	10	8	2	3	folic	Ly Eos*	0
14	DRESS	74806	DS/DR	4	6	1	1	4	acr-syr	Ly	0
15	DRESS	74027	DS	4	5	4	3	0		Ly	6
16	DRESS	73110a	DS	4	7	3	1	0		Ly	0
		73110b	DS/DR	4	7	6	2	1	peri-neural	Ly	0
17	SJS	72982	DS	6	6	6	1	0		Ly	0
18	TEN	66661	DS	10	8	9	6	2	peri-eccrine	Ly Eos*	8
%				15/18 83.3%	18/18 100%	15/18 83.3%	14/18 77.7%	9/18 50%		L-100% N-16.6% E-22.2%	44.4%

DS/DR/DD: dermis superficialis, dermis reticularis, deep dermis:
DEJ: dermal-epidermal junction;
RBC: red blood cells; Ly: lymphocytes, Neut: neutrophils, Eos: eosinophils;
folic: follicular; acr-syr: acrosyringeal
* eosinophils mainly in the interstitial dermis

Table E.5 – Mean scores for epidermal (a) and dermal (b) changes and their frequency (%) in patch tests from the different clinical patterns of CADR

a)

clinical pattern	epidermal changes											
	spongiosis		exocytosis		vesicles		pustules		necrosis		vacuolization keratinocytes	
AGEP	3.5 ±0.6	100%	4.2 ±1.8	100%	0	0%	4.6 ±3.7	80%	0	0%	0.4 ±0.9	20%
MPE	5 ±2.1	100%	5.3 ±2.1	100%	1.7 ±2.3	67%	1.3 ±2.1	33%	0	0%	2.8 ±2.9	100%
DRESS	7.4 ±3.8	100%	6.0 ±2.5	100%	9.2 ±4.4	100%	0	0%	1.3 ±2.5	33%	2.8 ±2.2	80%
SJS/TEN	5.5 ±3.5	100%	7.0 ±1.4	100%	5.5 ±3.5	100%	0	0%	6.0 ±6.0	50%	6.5 ±3.5	100%

b)

clinical pattern	dermal changes									
	edema		extravascular RBC		inflammatory infiltrate					
					perivascular		interstitial		DEJ	
AGEP	2.6 ±1.7	80%	0	0%	4.2 ±1.8	100%	2.4 ±2.3	60%	0.4 ±0.9	20%
MPE	2.2 ±2.2	67%	1.7 ±2.2	67%	6.8 ±1.5	100%	3.7 ±2.1	83%	2.3 ±0.8	67%
DRESS	3.8 ±1.8	100%	3.2 ±4.6	40%	7.0 ±1.8	100%	4.2 ±2.9	100%	2.2 ±0.8	100%
SJS/TEN	8.0 ±2.8	100%	4.0 ±5.6	50%	7.0 ±1.4	100%	7.5 ±2.1	100%	3.5 ±3.5	50%

Table E.6 – Comparative scores for histological parameters in different drug patch tests, namely from amoxicillin (AMX) and other antibiotics and from carbamazepine (CBZ) and other anticonvulsants

Reactive drug on PT	epidermal changes					dermal changes			
	spongiosis	exocytosis	vesicles	pustules	vacuol. basal	edema	inflammatory infiltrate		
							perivasc	interstitial	DEJ
AMX	3.3	5.2	0.3	1.3	3.5	0.7	6.3	3.3	2.0
Antibiotics	4.6	5.2	1.3	1.9	2.1	2.1	5.7	3.1	2.0
CBZ	5.7	7.3	10.6	0	4.3	5	6.6	5.0	0.7
Anticonvulsants	5.0	6.2	8.5	0	3.7	4.75	6.5	4	0.7

E.5.3. Immunohistochemistry of positive patch tests

An immunohistochemistry study with T cell phenotypic markers was performed in the 2 cases with the denser infiltrate (pts. 13 and 18).

The patch test from TEN revealed a very dense lymphocyte infiltrate in the upper and mid dermis, that was composed mainly of $CD3^+$ T cells, predominantly $CD8^+$ T cells. Some cells were expressing the cytotoxic marker $CD56^+$. Both $CD8^+$ and $CD56^+$ cells were also observed in the epidermis (Fig. E.8). A few cells of the infiltrate were also stained by granzyme B (data not shown).

In the case of DRESS from abacavir, dermal and epidermal cells were also mainly $CD8^+$, but very few cells expressed granzyme B. There was a significant dermal accumulation of $CD8^+$ T cells below areas of more intense epidermal aggression, with vesicles and vacuolization. Some $CD8^+$ T cells in the epidermis were adjacent to necrotic keratinocytes (Fig. E.7).

E.5.4. Histopathology of patch tests in correlation with the pattern of cutaneous adverse drug reaction

In the 4 out of 5 patch tests from AGEP, we observed mainly intraepidermal or subcorneal spongiform pustules with neutrophil and/or lymphocyte exocytosis, which was very similar to the histopathology of the acute CADR, as shown in patient 1 (Fig. E.3). Moderate spongiosis was observed in all cases, mostly without vesicles, necrotic keratinocytes or vacuolization of the basal layer. The dermal infiltrate was mostly perivascular, constituted mainly by lymphocytes and with associated neutrophils in 2 of the 5 cases (Fig. E.3 and E.4).

In the 6 cases of maculopapular exanthema, the most striking feature was the vacuolization of the basal epidermal layer, associated with spongiosis and lymphocyte exocytosis in all cases, but forming intraepidermal vesicles in only

in three cases. No necrotic keratinocytes were observed. There was a predominantly perivascular dermal infiltrate of lymphocytes, some also present at the dermal epidermal junction. Very occasionally, eosinophils or neutrophils could also be observed (Fig. E.5).

In the 5 cases of DRESS, there was an intense spongiosis with lymphocyte and/or a mixed cell exocytosis and formation of important intraepidermal vesicles in all cases. A denser perivascular or interstitial lymphocyte dermal infiltrate, and a less intense dermal-epidermal infiltrate, were observed in all cases, with a peridanexal infiltration also in half of them. In two cases esosinophils were present, both at the dermis and epidermis (Fig. E.6 and E.7).

The most significant characteristic of the patch test from a case of TEN was the epidermal necrosis that affected all the keratinocyte layers, observed in the large part of the skin biopsy. Marked subepidermal edema and large and mostly unilocular subcorneal vesicles with necrotic keratinocytes in its limits were also observed in a limit of the skin biopsy (Fig. E.8).

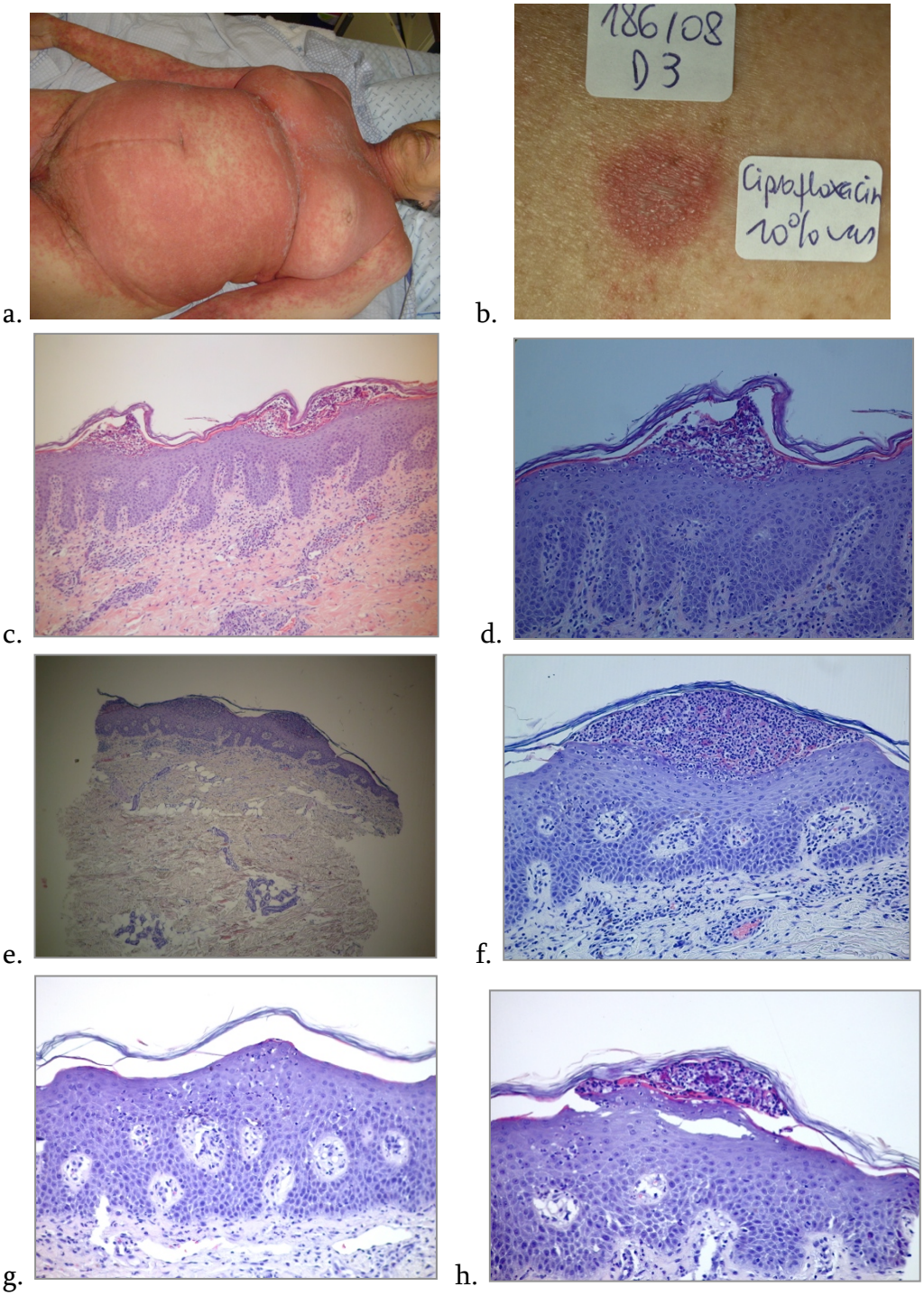


Figure E.3 – Images from patient 1 with psoriasis who developed AGEP from ciprofloxacin (a) and had a pustular patch test with ciprofloxacin and other fluorquinolones (b). There is a remarkable similarity between the intraepidermal/subcorneal spongiform pustules observed both during the acute episode (c,d) and in the patch test (e,f). Acanthosis (g) and intense neutrophil exocytosis towards a subcorneal pustule (h) were also observed in the patch test.

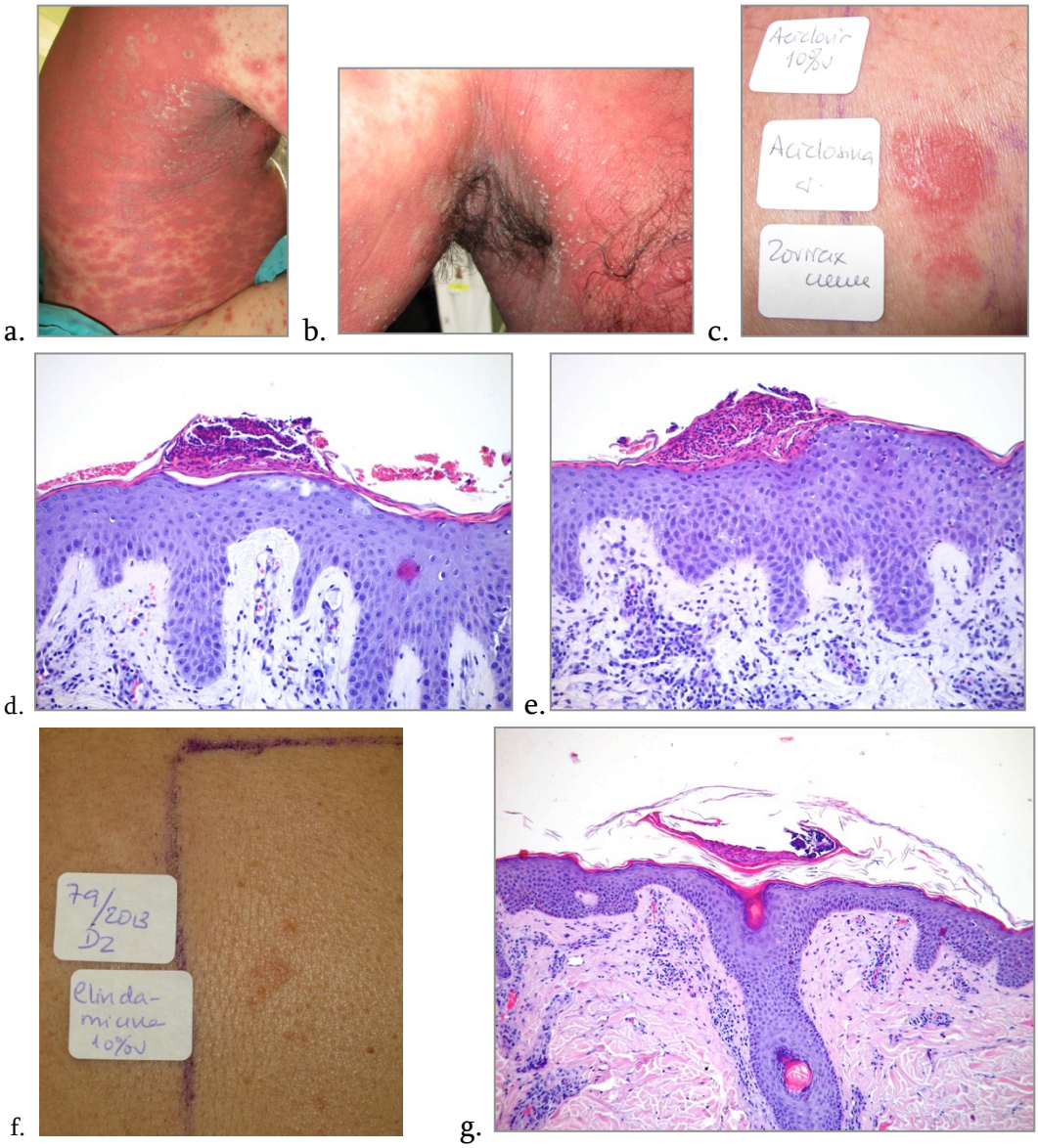


Figure E.4 - Pustular reactions in AGEP cases. Pt 2 with AGEP from acyclovir, observed during the acute eruption (a, b) and the pustular patch test to the commercial cream containing acyclovir (Aciclosina®) (c). A biopsy from the patch test showing several intracorneal or subcorneal pustules, with intense dermal edema, vasodilation, erythrocyte extravasation and intravascular lymphocytes eosinophils and neutrophils (d,e). The positive patch test in AGEP from clindamycin showing rare pustules (f) was associated with a follicular pustule in skin histopathology (g).

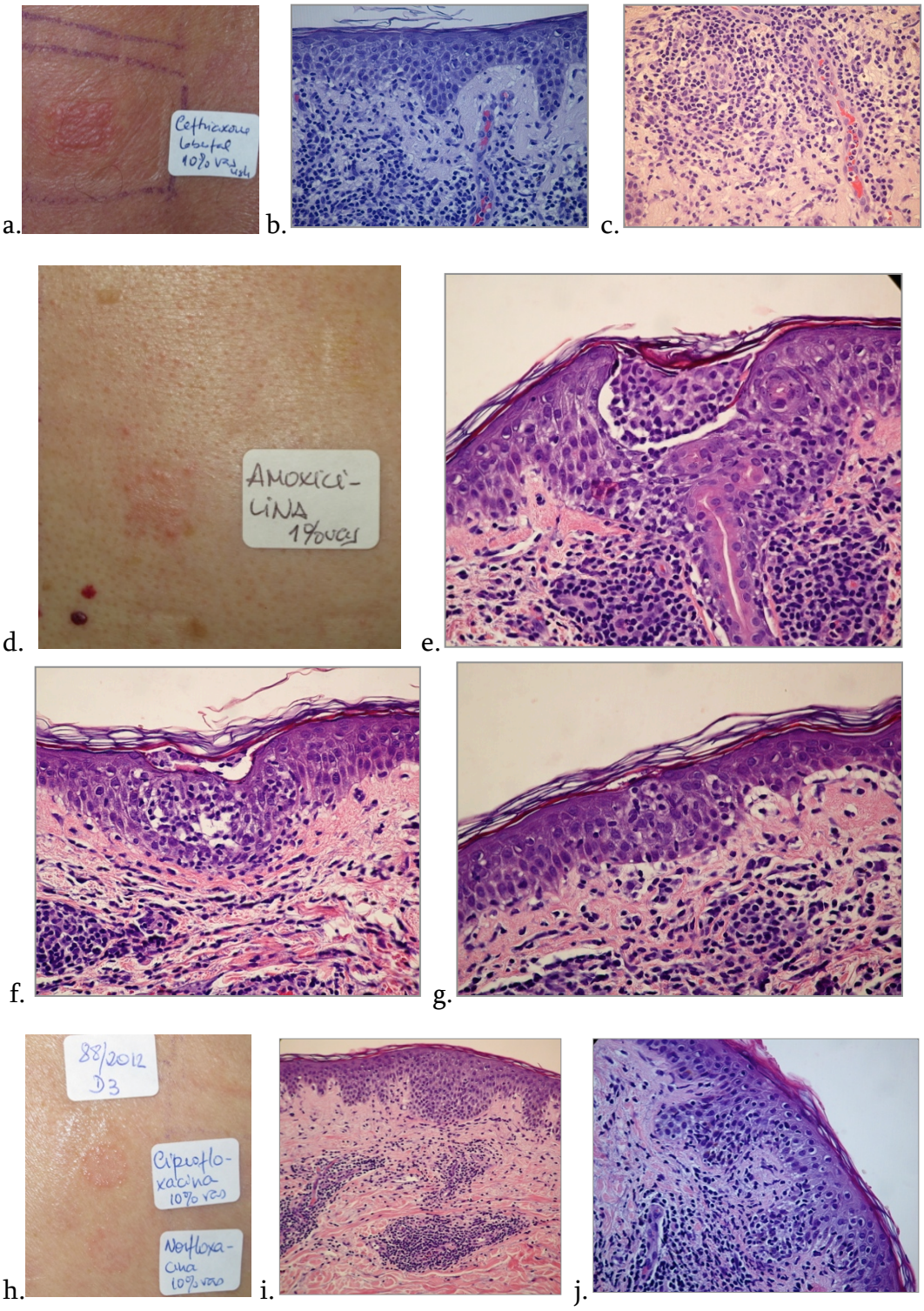
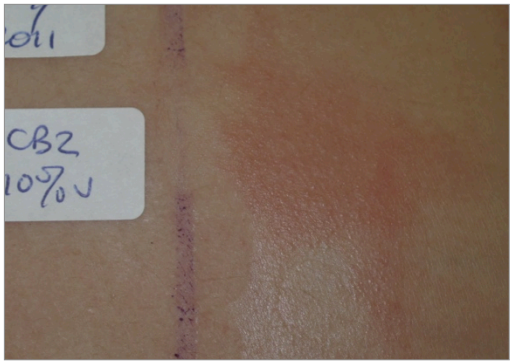


Figure E.5 – Positive patch tests in maculopapular exanthema in patients 6 (a-c), 7 (d-g) and 8 (h-j), with a significant vacuolization of basal cells (b,g,j), lymphocyte exocytosis forming small vesicles (f), or clusters of histiocytes (e,g), and a dense dermal infiltrate with a significant number of intravascular neutrophils (b,c).

a. 

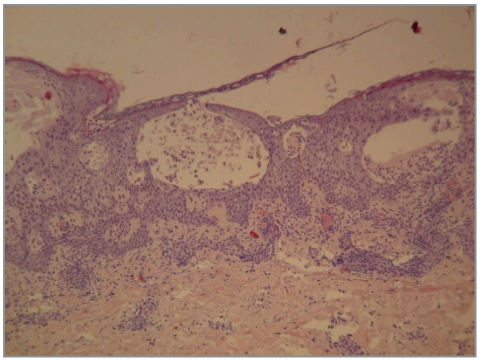
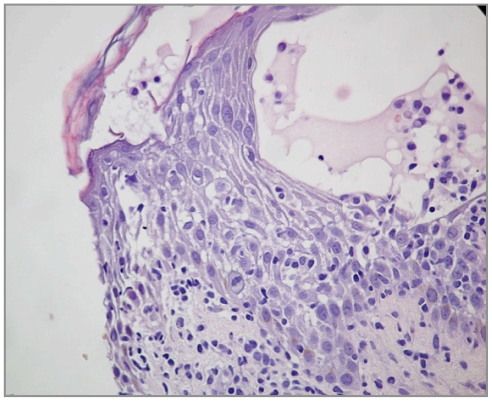
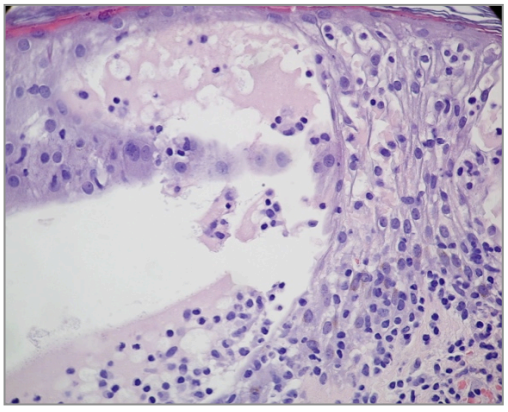
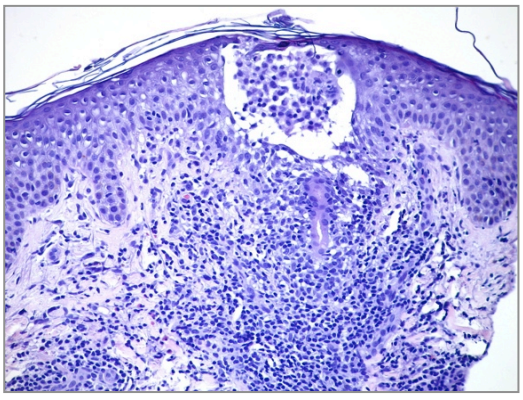
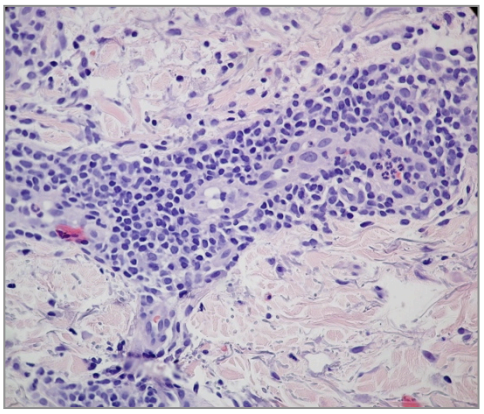
b. 
c. 
d. 
e. 
f. 

Figure E.6 – Positive patch test from carbamazepine in DRESS (pt 14). Histological aspects of the positive patch test with an intense epidermal aggression, with remarkable spongiosis and vesicle formation (b-d), with clusters of histiocytes within the vesicles (g) and a dense dermal inflammatory infiltrate with some esosinophils (f).

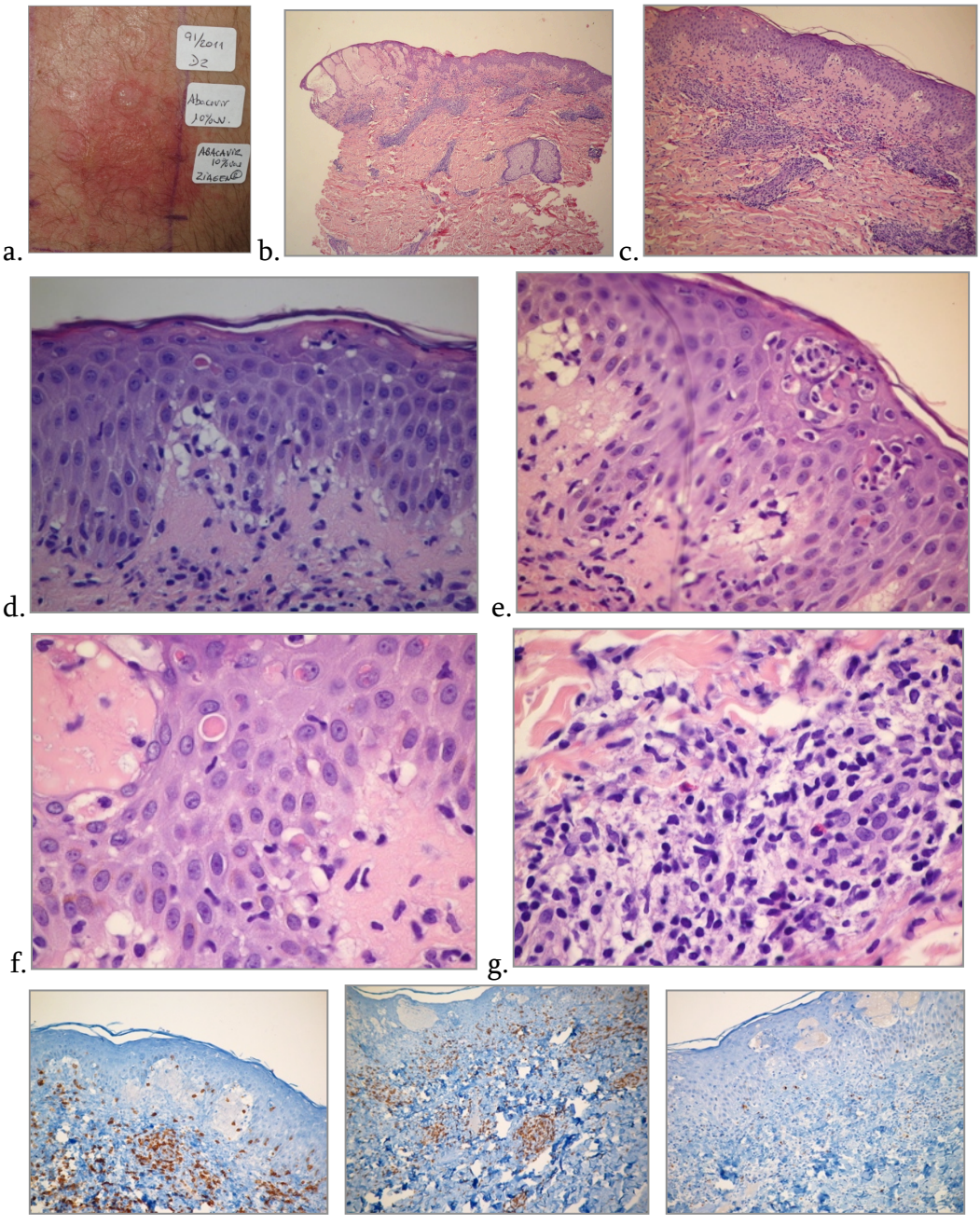


Figure E.7 – Positive patch tests in DRESS from abacavir (Ziagen®) in pt 13 (a). Large vesicles in one extreme of the patch test and a dense, mainly perivascular lymphocyte infiltrate in the upper and mid-dermis (c), with no atypical cells, but with many eosinophils (g), although there was no eosinophilia during the acute phase. Significant epidermal aggression with vacuolization of basal cells (d), isolated necrotic keratinocytes (e,f) and exocytosis of lymphocytes forming microabscesses (e). Immunohistochemistry showed a significant presence of $CD8^+$ T cells in the infiltrate, mainly below areas of more intense epidermal involvement, with several $CD8^+$ T cells adjacent to necrotic keratinocytes. Staining for granzyme B was relatively poor.

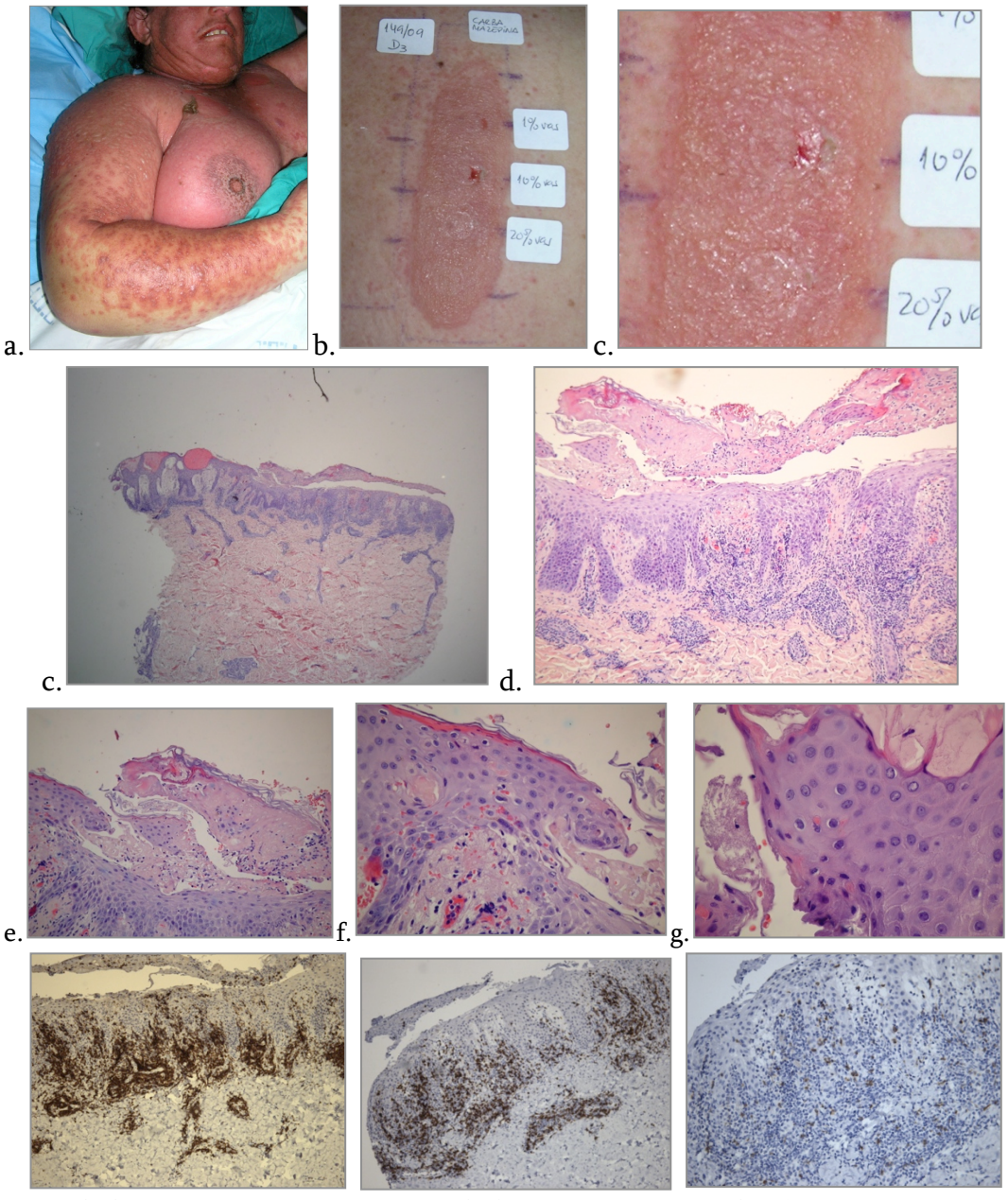
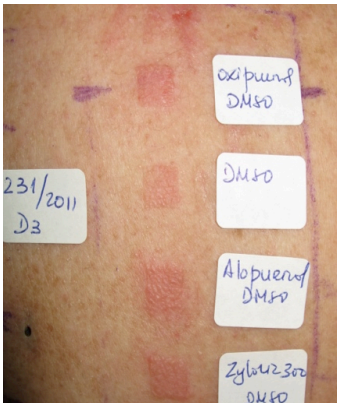
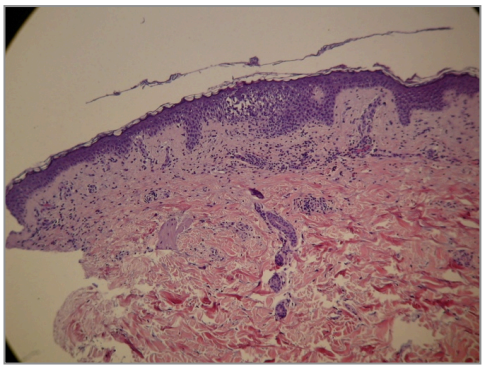
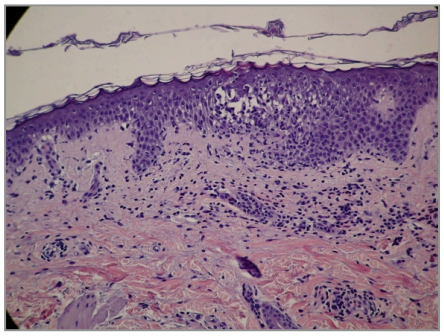
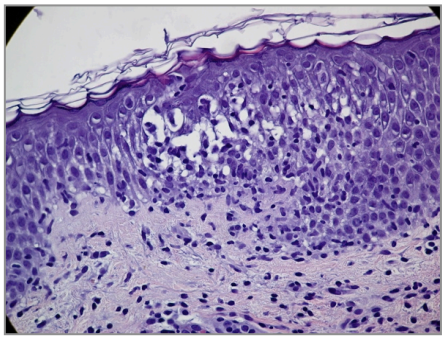


Figure E.8 – TEN from carbamazepine in pt 18 (a) with a patch test with confluent vesicles and epidermal necrosis with Nikolsky's sign (b). Histopathology showed vesicles (c) and, specially, keratinocyte necrosis involving the whole epidermal thickness (d). In certain areas, epidermal necrosis coexisted with isolated or confluent necrotic keratinocytes (e-g) and aspects suggesting satellite cell necrosis (f,g). Immunohistochemistry revealed a dense T cell infiltrate ($CD3^+$) (h) mainly $CD8^+$ in the dermis but also in the epidermis (i), with a reduced infiltration of $CD56^+$ cytotoxic cells (j).

a. 

b. 

c. 

d. 

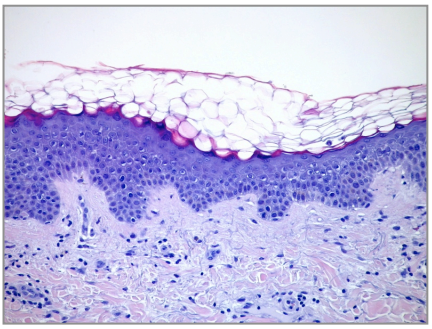
e. 

Figure E.9 – Pt 16 with DRESS from allopurinol with positive patch tests with allopurinol, the powder of Zyloric® pills and oxypurinol pre-dissolved in DMSO before incorporation in petrolatum, contrasting with a less intense reaction with DMSO alone (a). Histopathology of the patch with allopurinol, with lymphocyte infiltrate in the upper dermis and exocytosis forming vesicles (b-d), contrasts with a lesser infiltrate in the control patch test with DMSO (e). Both biopsies showed a spiky horny layer with a basket weave appearance (c-e) and a cytotoxic aspect of keratinocytes with shrinkage of the cytoplasm, which may be attributed to DMSO (e).

E.6. Discussion

Patch testing is usually performed in delayed CADR for identifying the culprit drug, but a skin biopsy is seldom performed. Therefore, apart from occasional case reports, most of them from AGEP, there are very few descriptions of the histopathology of patch tests in CADR.

In this study we included a group of 18 patients who had positive patch tests in the evaluation of delayed CADR with different clinical patterns (maculopapular exanthema, AGEP, DRESS and SJS/TEN) and induced by different drugs. Positive patch tests were mostly from carbamazepine, amoxicillin, fluorquinolones, clindamycin, abacavir and diltiazem, drugs that frequently induce positive patch tests (Barbaud 2005),(Gonçalo et al. 2006),(Santiago et al. 2010),(Rive et al. 2013),(Barbaud et al. 2013a). Moreover, we included patients with CADR patterns that most frequently induce positive reactions (maculopapular exanthema, AGEP and DRESS) and, also, 2 cases with SJS/TEN, where positive patch test reactions are not so frequently reported (Wolkenstein et al. 1996).

In general, patch tests showed features that simulated the acute eruption, both macroscopically and on histopathology.

E.6.1. Macroscopic features of positive patch tests

In this group of 18 patients, patch tests showed some particular macroscopic features apart from the erythema, infiltration and vesicles, usually observed in the study of ACD.

A marked edge effect was observed with fluorquinolones and acyclovir containing creams, which is, nevertheless, similar to the observations described also in some patch tests in the study of contact allergy, particularly from topical corticosteroids (Isaksson et al. 1999).

Macroscopic pustules, observed mainly in AGEP cases, are also occasionally observed in patch tests from contact allergens, particularly from nickel and other metals. Although initially considered irritant, pustules are now explained by the capacity of Ni-specific T cells to produce high amounts of IL-17 (Pennino et al. 2010),(Dyring-Andersen et al. 2013). This cytokine has a potent effect on activation of the innate immunity on keratinocytes. It facilitates T-cell adhesiveness through ICAM-1 and, consequently, T-cell-induced keratinocyte killing. Moreover, IL-17 enhances keratinocyte secretion of IL-8/CXCL8, which is a potent chemoattractant for neutrophils (Pennino et al. 2010), and explains the accumulation of neutrophils in the epidermis and the frequent observation of pustules in nickel patch tests. A similar mechanism, involving drug specific Th17 cells and IL-8/CXCL8, is considered essential in the pathophysiology of AGEP (Kabashima et al. 2011). Therefore, TH17 and IL-8/CXCL8 may justify the observation of pustules in the positive patch tests in our AGEP patients, and also in many previous reports (Britschgi et al. 2001),(Britschgi and Pichler 2002),(Barbaud 2009),(Serra et al. 2011),(Serra et al. 2012).

Interestingly, in the positive patch test from TEN, we observed an area of vesicle confluence or skin necrosis that lead to the Nikolsky's sign, a typical sign observed in TEN, for which we found no similar reference in the few cases of positive patch tests in this CADR.

In general, the different macroscopic aspects of the patch tests, observed in CADR, with some similarity to the pattern of lesions observed during the acute eruption, are comparable to the clinical variability of allergic contact reactions, that may present under different clinical patterns (papular, vesicular, lichenoid, pigmented, bullous, erythema-multiforme-like or lymphomatoid-like) with some correspondent macroscopic variations in the respective patch tests (Goon and Goh 2011).

E.6.2. Histopathology of patch tests with drugs

On histopathology, we observed aspects in positive patch tests that are similar to those usually described in the acute CADR. Unfortunately, we do not have skin biopsies from most of the acute CADR in these patients to compare histopathology, therefore, we report to the histopathology usually described in the corresponding CADR patterns.

The main histopathologic changes observed in patch tests from CADR, namely spongiosis, lymphocyte exocytosis forming vesicles, a dermal inflammatory infiltrate with perivascular and interstitial lymphocytes, occasional eosinophils and extravasation of erythrocytes, is also the pattern usually described in patch tests with contact allergens (Lachapelle and Marot 2011).

The different histopathologic aspects, observed in the patch tests from CADR in our patients, are comparable to the variability in the histopathology of the distinct clinical patterns of contact allergic reactions. Pustular and neutrophilic reactions, a more severe degree of epidermal necrosis, like in erythema multiforme, a band-like infiltrate with basal cell vacuolization or, even, a dense infiltrate simulating a cutaneous lymphoma, have all been described in ACD (Goon and Goh 2011). This seems to reflect the involvement of different sub-phenotypes of allergen specific effector T cells in ACD (Rustemeyer et al. 2011).

Similarly, in CADR, drug specific T cells belong to distinct sub-phenotypes that differently orchestrate the immuno-inflammatory effector skin reaction, inducing different clinical reactions patterns (Pichler 2007),(Pichler et al. 2011),(Adam et al. 2011),(Rozières et al. 2009b). As drug specific T cells isolated from positive patch tests have functional characteristics similar to those isolated from the skin during the acute eruption (Yawalkar et al. 2000),(Kuechler et al. 2004), it may be expected that patch tests also reflect, macro and microscopically, the variability in effector function of the different sub-phenotypes of T cells. This supports our findings that histopathologic changes mimic the histologic patterns usually observed in the corresponding

type of CADR. This is extremely important, as it allows the use of patch testing to better characterize the sub-phenotypes of T cells involved in each CADR and, also, to understand, effector pathomechanisms involved in each CADR.

E.6.2.1. Patch tests in acute generalized exanthematous pustulosis

In AGEP, skin biopsies showed intraepidermal pustules, with a spongiform pattern, that, at the time of biopsy, were localized within the horny layer or just below it, as during the acute CADR. In 2 cases, we observed mostly neutrophilic exocytosis but, in the other cases, lymphocytes were the main cell in exocytosis and, also, in the dermal inflammatory infiltrate, which is in agreement with the present understanding of the pathophysiology of AGEP.

Drug specific T cells, $CD8^+$, $CD4^+$ or Th17, are considered the main effector cells in AGEP. They infiltrate the skin during the CADR, probably also in the patch test, and produce high amounts of IL-8/CXCL8. This chemokine, directly, or indirectly through IL-17 and IL-22, attracts neutrophils to the epidermis and, along with GM-CSF, enhances epidermal survival of neutrophils promoting the formation of pustules (Sidoroff et al. 2001),(Sidoroff 2012),(Halevy 2009),(Kabashima et al. 2011).

E.6.2.2. Patch tests in maculopapular exanthema

In patch tests from maculopapular exanthema we observed, in common with patch tests from the other CADRs, a lymphocyte perivascular and interstitial dermal infiltrate, spongiosis, and exocytosis. This type of epidermal and dermal reaction is very similar to the histopathologic changes of maculopapular exanthema, described in one of the most extensive studies performed early during the course of a maculopapular exanthema, which included 60 biopsies

from 48 patients (Naim et al. 2011). Also, in another study with 35 cases with a proven drug-induced maculopapular exanthema, a spongiotic and interface or a perivascular pattern, represented the main observations (Seitz et al. 2013).

A very characteristic aspect, observed in all the biopsies of the patch tests from maculopapular exanthema, was the vacuolization of the keratinocytes in the basal layer. A similar aspect was also observed in 97% of the cases of maculopapular exanthema in the study by Naim and co-workers (Naim et al. 2011) and this may also fit into the spongiotic and vacuolar interface dermatitis described by Seitz and co-workers (Seitz et al. 2013). Dermal edema, extravascular erythrocytes and neutrophils in the lumina of blood vessels, observed by us respectively in 67%, 67% and 100% of the cases, were also frequent in Naim's study, respectively in 85%, 28% and 38% of their 60 biopsies (Naim et al. 2011), and, also, in the more recent study (Seitz et al. 2013).

On the contrary, we found no necrotic keratinocytes, reported in 22% of Naim's cases and by one observer of Seitz' study (Naim et al. 2011), (Seitz et al. 2013). This may also be explained as, in their study, Naim and co-workers may have included some cases of exanthema associated with DRESS, as they refer to cases that lasted 56 days, which is not typical of a simple maculopapular exanthema.

Eosinophils or neutrophils were scarce and occurred in few of our cases, compared to their presence in more than 55-77% of Naim's cases. It is, nevertheless, interesting to note that, in patch tests, like in the acute eruption also, eosinophils can be completely absent (or not detected on haematoxylin-eosin staining), although they are generally considered a marker of drug-induced allergic skin reactions. Also, no difference in eosinophil infiltration was observed between a proven drug-induced exanthema and an exanthema by other possible causes (Seitz et al. 2013)

E.6.2.3. Patch tests in DRESS

In DRESS, we observed aspects similar to the patch tests in maculopapular exanthema, although associated with some scattered necrotic keratinocytes, more intense spongiosis and frequent formation of vesicles. This may reflect a higher aggressive capacity of T cells in DRESS than in maculopapular exanthema, causing also, usually, a more severe and long lasting exanthema (Descamps and Ranger-Rogez 2014).

Necrotic keratinocytes, observed in occasional patch tests in DRESS, are also found in acute lesions. Nevertheless, as we previously noticed, spongiosis, frequently found in the acute exanthema, is not usually associated with vesicles. The significant presence of vesicles in the patch test may reflect the direct application of the drug in the skin, during the patch test, which my combine with skin cells in higher concentrations and, therefore, promote more epidermal inflammation. Moreover, if we consider that, during the patch test, the dermal anti-viral T cell response is probably absent, or scarce, we may admit that most of the T cell response will be directed towards epidermal cells that bind the drug.

But, globally, there is a significant similarity of histopathology findings in patch tests in DRESS and maculopapular exanthema, particularly when considering the vacuolization of the basal epidermal cells, exocytosis and the intensity of the perivascular and dermal-epidermal infiltrate. This similarity is also reproduced in histopathology during the acute reactions, in DRESS and maculopapular exanthema. Therefore, similarity is striking when comparing results from patch testing in the two types of CADR and the results presented in the previous chapter and those described by Naim *et al.* in their study of acute maculopapular exanthema (Naim et al. 2011). These similarities at the histopathology level may reinforce the idea of a possible continuous spectrum between DRESS and maculopapular exanthema, which is also suggested from the clinical presentation of these two CADR. Moreover, similar findings in

patch tests and the acute eruption may reinforce the use of patch testing to study effector pathomechanisms in DRESS, although probably, in this case, the contribution of viral reactivation will be minor.

Eosinophilia, a hallmark of DRESS during the acute phase, is not associated with a prominent skin infiltration by eosinophils, neither during the acute eruption nor in the patch tests. Nevertheless, we observed eosinophil exocytosis in 2 patch tests and eosinophils in the interstitial dermis in another patch test.

In DRESS from abacavir, the positive patch test was very rich in T cells in the dermis and in exocytosis, with some lymphocytes forming epidermal microabscesses, as usually reported in cutaneous T cell lymphoma. Nevertheless, lymphocytes were not atypical or in mitosis, as we observed in the case of pseudolymphomatous reaction in the acute exanthema in DRESS (pt. 6, chapter D) or as has been reported in DRESS induced by anticonsulsants with a prominent pseudolymphomatous infiltration (Husain et al. 2013).

In this case of DRESS from abacavir, there was an important contribution of $CD8^+$ T cells, some migrating to the epidermis where they localized around necrotic keratinocytes. This is in agreement with the participation of $CD8^+$ cytotoxic T cells in abacavir hypersensitivity, as these cells recognize the drug in association with HLA-B*57:01 (Rive et al. 2013),(Bell et al. 2013), the class I HLA haplotype also present in our patient.

E.6.2.4. Patch tests in Stevens-Johnson syndrome/Toxic epidermal necrolysis

The positive patch test in a case of TEN induced by carbamazepine, a rare case of a positive patch test in TEN (Wolkenstein et al. 1996), is a very good example of the histological similarity between the acute CADR and the positive

patch test. Although very well formed vesicles could be seen in an extremity of the biopsy, in the most affected area of the patch test there was necrosis of the full thickness of the epidermis, which is the hallmark of TEN (Quinn et al. 2005),(Valeyrie-Allanore et al. 2013).

Lymphocyte exocytosis was intense, occasionally with these cells surrounding necrotic keratinocytes, resembling satellite cell necrosis. This aspect was originally described in the acute bullous eruption of graft-versus-host disease (GVHD), which is clinically very similar to TEN. In GVHD, lymphocytes, received from the donor, recognize host keratinocytes carrying a different HLA, and induce necrosis by direct cell contact (Piérard et al. 2013).

In the patch test reaction of our TEN patient, we observed, mainly in the dermis, but also in the epidermis, abundant cytotoxic $CD8^+$ T cells, which are considered one of the main effector T cells in TEN. Some are very probably drug specific and recognize and kill keratinocytes expressing HLA combined with the drug (Roujeau et al. 2011), which can justify the observation of the images suggesting satellite-cell necrosis in the patch test.

Nevertheless, at present, it is believed that the main mechanism of keratinocyte death in TEN does not depend on direct contact between the cytotoxic T cell and the keratinocyte, and this explains why, in our patch test, there were only occasional cytotoxic $CD8^+$ and $CD56^+$ cells in the epidermis in contrast with the extensive epidermal necrosis. In TEN, keratinocyte death is mainly mediated by soluble lymphocytic factors that may exert their necrolytic/apoptotic effect at a distance, like granulysin, FasL, TNF-α and other TNF related death molecules (Chung et al. 2008)(Viard-Leveugle et al. 2013),(Schwartz et al. 2013a). Moreover, drug specific T cells are most often found in the blister fluid of TEN (Correia et al. 1993),(Chung and Hung 2012) or in the dermis, where they were preferentially observed in our patch test.

E.6.3. Patch tests with allopurinol and oxypurinol

Allopurinol, even tested in higher concentrations (20%) and after tape-stripping, seldom induces positive patch tests in petrolatum or in different vehicles that have been tried (Vieira et al. 2004b),(Santiago et al. 2010). A similar situation occurs with oxypurinol, its active metabolite that lasts longer in circulation and is considered responsible for the delayed immune reaction, as it has been shown to stimulate specific T cells *in vitro* (Hamanaka et al. 1998),(Yun et al. 2012b),(Yun et al. 2014).

Both chemicals, allopurinol and oxypurinol, have a molecular weight adequate for inducing epicutaneous patch tests reactions, respectively 136.11 and 152.11g/mol, but they are highly insoluble. Therefore, we tried a new vehicle using dimethyl sulfoxide to solubilize them and enhance their skin penetration. At present, controls tested also with allopurinol and oxypurinol in this vehicle were negative or had very a faint irritant reaction that we attributed to DMSO (data on file).

In this study, positive reactions were observed in 2 patients (DRESS and SJS), both with allopurinol, oxypurinol and, also, with DMSO alone, whose test was also studied with a skin biopsy. In both cases the patch test reaction with allopurinol or oxypurinol was more intense than the vehicle alone. In histology, the scores for basal cell vacuolization, exocytosis, vesicle formation and intensity of the dermal infiltrate were slightly higher in the biopsy from the patch test with allopurinol, although not particularly different from the vehicle (Table E.3 and E.4 and Fig. E.9). Therefore, this does not allows us to take significant conclusions on the utility of this solvent for patch testing patients with suspected allopurinol induced CADR.

In skin biopsies in these tests, we observed a very particular aspect of the horny layer and outer epidermal layers, namely a spiky horny layer with a basket weave appearance of the outer corneal layer, shrinkage of the cytoplasm of keratinocytes and a dermal and epidermal lymphocyte inflammatory infiltrate. These changes can be attributed to the powerful solvent effect and keratinocyte

aggression induced by DMSO, with a consequent inflammatory infiltrate, as observed in studies of irritant contact dermatitis induced by this potent solvent (Sjögren and Anderson 2000).

E.6.4. Patch tests in the study of cross reactions

Cross-reactions determined at the patch test level were observed in 8 patients. They may be relevant for the patient, but they were not studied on histology, as this was not the main objective of our study.

Cross-reactions were observed between different fluorquinolones (2 cases) and different betalactam antibiotics (6 cases).

As usually reported, cross-reactions between betalactams occur constantly between ampicillin and amoxicillin (4 cases), and less often between aminopenicillins and other penicillins, such as dicloxacillin (pt. 11). As cephalosporins have a different belactam ring, cross-reactivity with penicillins occurs, only very occasionally, between aminopenicillins and some cephalosporins with a similar R2 amino side chain, such as cephalexin, cefradoxil, cefaclor, cefotaxim and ceftriaxone (Romano et al. 2004),(Romano et al. 2005),(Romano et al. 2012),(Romano and Caubet 2014), which may explain patch test results observed in patient 11. Concerning carbapenems, that have a similar betalactam ring but a different side chain, cross reactivity with benzylpenicillin or aminopenicillins is exceptional, both at patch test level as well as on drug provocation tests, according to the studies of Romano and collaborators (Romano et al. 2013), and our personal experience.

In other published series, patch tests were shown to be important to recognize cross-reactions between pristinamycin and other synergistines (Barbaud et al. 2004), ephedrine and pseudoephedrine in exanthematous reactions (Barbaud 2007), and between piroxicam and tenoxicam, and hydroxyzine and related anti-histamines in fixed drug eruption (Oliveira et al. 1999),(Gonçalo et al.

2002),(Cravo et al. 2007). On the contrary, absence of cross-reaction at the patch test level could also be confirmed between etoricoxib and celecoxib in fixed drug eruption (Andrade and Gonçalo 2011) or tetrazepam and diazepam in delayed exanthematous eruptions (Pirker et al. 2002),(Barbaud et al. 2009),(Thomas et al. 2008).

Interestingly, these patterns of cross reactivity may vary with the pattern of CADR. Cross-reaction between oxicams, frequently described in fixed drug eruption (Andrade et al. 2011), and cross-reactions between fluorquinolones observed in patients 1 and 8, respectively with AGEP and maculopapular exanthema, are not usually observed when the CADR pattern is photosensitivity (Gonçalo et al. 1992),(Oliveira et al. 1996). This can be explained by the formation of a new photoproduct that is antigenically different, as was shown for piroxicam that, upon UVA irradiation, gives rise to a chemical that is antigenically and structurally similar to thiosalicylic acid whereas the other oxicams do not form such a photoproduct on irradiation (Figueiredo 1994),(Gonçalo et al. 2002). Moreover, the absence of cross-reactions between benzodiazepines in CADR, contrasts with a very high frequency of cross-reactions between tetrazepam and several benzodiazepines, observed in patients with ACD due to airborne exposure to the powder of these drugs, particularly in nurses who have to crush tablets to give their patients (Vander Hulst et al. 2010), (Landeck et al. 2012),(Swinnen et al. 2014).

E.7. Conclusions

Patch testing was shown to be a useful tool in confirming the culprit drug involved in CADR and in studying cross-reactions that are very relevant for patient orientation. Unhappily, for reasons that are not yet completely understood, with some drugs and in some CADR patterns, the skin reaction cannot be elicited by the epicutaneous drug exposure, as in the patch test. Absence of concomitant stimulating factors, which are present only during the

acute eruption, may be one explanation. Problems of epidermal penetration or insufficient skin metabolism of the drug into the reactive metabolite may be another cause. In other cases, patch tests will be positive only in limited areas where previous lesions have occurred, as in some exanthema or in fixed drug eruption (Barbaud et al. 2001b),(Alanko 1994),(Gonçalo et al. 2002),(Andrade et al. 2011), as resting drug specific T cells are exclusively present in that area, and for a long time (Shiohara et al. 2002). In skin areas where resting drug specific T cells do not exist in enough amounts, we may assume that the drug needs to induce an innate immune response and activate antigen presenting cells before they attract drug specific T cells and induce the inflammation responsible for the positive patch test.

Although we have studied a reduced number of patch test reactions, and a very limited number in each clinical pattern of CADR, we have shown that a positive patch test in a delayed CADR reproduces partially the clinical and histopathologic pattern of the corresponding CADR.

As drug specific T cells isolated from positive patch tests have a phenotype similar to the one found in the blood or on the skin during the acute eruption (Yawalkar et al. 2000), we may expect that these T cells also induce the same aggressive pattern when the drug is delivered through the epidermis, as in a patch test. Therefore, with some limitations on the final extrapolation, patch testing can be used to study the main cells involved in the reaction, their phenotype and aggressive machinery and evaluate how the cells use of their effector functions during the skin inflammation, in the patch tested skin. This may significantly contribute to a better understanding of the pathophysiologic mechanisms involved in delayed CADR.

F.
CLINICAL STUDIES.
III - HLA studies in severe cutaneous adverse drug reactions from ALLOPURINOL

F. CLINICAL STUDIES. III - HLA STUDIES IN SEVERE CUTANEOUS ADVERSE DRUG REACTIONS FROM ALLOPURINOL[3]

F.1. Introduction

Allopurinol, widely used to lower uric acid levels, is considered usually well tolerated, but it has been associated with CADR in 2-3% of exposed individuals. All patterns of CADR involving delayed hypersensitivity mechanisms have been described with allopurinol, namely maculopapular exanthema, SJS/TEN, DRESS (Santiago et al. 2010),(Mockenhaupt 2012) and, less frequently, fixed drug eruption (Kim et al. 2012) or AGEP (Atzori et al. 2012),(Teo et al. 2011).

Allopurinol-induced CADR are often severe. Fatal cases of allopurinol-induced CADR are reported to occur in up to 0.4% of exposed individuals (Hershfield et al. 2013),(Kim et al. 2013). In several studies worldwide, allopurinol is considered is one of the most frequent causes of severe CADR (Halevy et al. 2008),(Mockenhaupt 2012),(Huang et al. 2011),(Lee et al. 2012b),(Atzori et al. 2012),(Pinheiro et al. 2013).

In studies conducted at the Department of Dermatology of the University Hospital of Coimbra most CADR requiring hospitalization, especially the severe ones, were attributed to allopurinol with a very high probability. In a

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[3] The results of this chapter have been published as:
HLA-B*58:01 is a risk factor for allopurinol induced DRESS and SJS/TEN in a Portuguese population. Margarida Gonçalo, I Coutinho, V Teixeira, AR Gameiro, MM Brites, R Nunes, A Martinho. *Brit J Dermatol* 2013: 169: 660-5. doi:10.1111/bjd.12389. (impact factor: 3.67),
and the full paper is included in this chapter with an additional introduction and final concluding remarks based on the new knowledge of this subject.

10-year study conducted between 2000 and 2010, allopurinol was responsible for 19 cases of SJS/TEN, representing 28.9% of all drug-induced cases. Moreover, it was associated with a higher mortality rate (18.2%) compared to the whole group of SJS/TEN evaluated in the same period (Brinca et al. 2011). In a 5-year survey conducted between 2003 and 2007, 19 among 42 cases of DRESS (45.2%) were imputed to allopurinol, used mostly for asymptomatic hyperuricemia (Santiago et al. 2008). In a more recent survey, conducted between 2008 and 2012, allopurinol was responsible for 48 of the 223 CADR hospitalized in our ward (21.5%). It was particularly responsible for severe reactions, e.g. 22 of the 49 cases of SJS/TEN and DRESS (44.9%) and 9 of the 19 cases of erythroderma or maculopapular exanthema with minor systemic symptoms that, nevertheless, did not fulfil the criteria for DRESS (47.4%) (Gouveia et al. 2013). In many of these cases allopurinol was used for asymptomatic hyperuricemia and at the highest dose (300mg/day) from the beginning of therapy, independent of the pre-existence of some degree of renal failure.

The frequency and severity of these reactions, with a significant patient suffering during and after the acute episode, was a strong motivation to perform the present study, exclusively with this drug. It was our objective to evaluate if any of the risk factors reported in other populations, namely the HLA-B*58:01, was also evident in our population. If positive and with a significant risk, this might be a strong argument to implement HLA pre-testing before initiating therapy and, therefore, avoid many of the challenging cases we regularly face in our Dermatology ward.

Moreover, as there are few European studies on the relation between HLA-B*58:01 and allopurinol and they only evaluate SJS/TEN patients, it was our intention to understand whether this relation was reproducible in another population of European ancestry, the Portuguese population, and if it was extensive to other patterns of severe CADR, namely DRESS, and, eventually, also to other less severe patterns of CADR induced by allopurinol, as maculopapular exanthema.

A strong risk association with a class-I HLA molecule and a CADR induced by allopurinol would also be an important element to contribute to understand the pathomechanisms involved in these CADR.

The analogy between delayed CADR induced by allopurinol and similar patterns of delayed CADR induced by other drugs, suggests that drug-reactive T cells are responsible for the specificity of the reaction (Gonçalo and Bruynzeel 2012), but patch testing with delayed readings and *in vitro* lymphocyte proliferation or stimulation tests, using both allopurinol and its metabolite oxypurinol, are usually negative (Vieira et al. 2004a),(Santiago et al. 2010),(Kim et al. 2012),(Hamanaka et al. 1998).

Association between HLA-B*58:01 and allopurinol is a strong argument in favour of the participation of drug specific $CD8^+$ T cells, as these cells recognize antigens coupled with HLA-I molecules (Yun et al. 2012a),(Bharadway et al. 2012),(Chung and Hung 2012). Most recent work strongly suggests a major contribution of $CD8^+$ T cells in delayed CADR from many other drugs, namely amoxicillin, abacavir and carbamazepine (Rozières et al. 2009b),(Rozières et al. 2010), (Phillips and Mallal 2009a),(Wei et al. 2012a),(Chessman et al. 2008). Moreover, $CD8^+$ T cells are important effector T cells and recognize the drug coupled with skin cells in many patterns of CADR, namely maculopapular exanthema (Schlapbach et al. 2011b), DRESS (Saito et al. 2012) and, very particularly, in SJS/TEN and fixed drug eruption (Chung et al. 2008).

At the time our study was conducted, allopurinol or oxypurinol specific $CD8^+$ T cells had not been isolated from the skin or blood in patients with allopurinol-induced CADR, but allopurinol/oxypurinol specific T cells had been generated *in vitro*, preferably using cells from HLA-B*58:01 donors (Yun et al. 2012b),(Lin et al. 2012). This was a strong argument that reinforced the participation of this HLA molecule on the specific drug recognition of allopurinol/oxypurinol by T cells and a confirmation of the involvement of delayed hypersensitivity mechanisms, also in the allopurinol-induced CADR.

In 2013 and 2014, oxypurinol and allopurinol reactive T cells clones generated from HLA-B*58:01 positive and negative donors were studied more deeply. Particularly from HLA-B*58:01 positive patients with CADR from allopurinol, reactive T cell clones have a higher affinity for oxypurinol than allopurinol, but HLA-B*58:01 is not absolutely needed for generating oxypurinol or allopurinol reactive clones. Interestingly, isolated oxypurinol reactive T cells clones are stimulated by oxypurinol in a dose dependent way, which is concordant with a clinical relation of CADR occurring preferentially with higher allopurinol levels, either by using higher therapeutic doses or due to renal insufficiency that reduces drug clearing (Yun et al. 2013). More recently, Yun and co-workers have shown that oxypurinol binds the F-pocket of the peptide binding groove of HLA-B*58:01, with more affinity than allopurinol. The oxygen at the position 6 of the pyrimidine ring in oxypurinol, that is absent in allopurinol, facilitates interaction with arginine 97 of the HLA molecule (Yun et al. 2014).

F.2. HLA-B*58:01 as a risk factor for allopurinol induced DRESS and SJS/TEN in a Portuguese population

THERAPEUTICS

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*HLA-B*58:01* is a risk factor for allopurinol-induced DRESS and Stevens–Johnson syndrome/toxic epidermal necrolysis in a Portuguese population

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Summary

Background *HLA-B*58:01* is associated with allopurinol-induced severe cutaneous adverse drug reactions (sCADR) particularly in Han Chinese, but the risk in European populations has seldom been studied.
Objective To study the association of *HLA-B*58:01* with allopurinol-induced sCADR in a Portuguese population.
Methods We studied 25 patients (11 male/14 female, mean age 67·4 years) with sCARD from allopurinol: 19 DRESS (drug reaction eosinophilia and systemic symptoms) and six Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN). *HLA* was genotyped by reverse sequence-specific oligonucleotide–polymerase chain reaction and results compared statistically with a control group of 23 allopurinol-tolerant individuals and the control population.
Results *HLA-B*58:01* was present in 16 patients with sCADR (64%) [12 DRESS (63%), four SJS/TEN (67%)], one allopurinol-tolerant individual (4%) and 63 normal controls (1·96%), with a statistically significant difference between sCADR and the two control groups. When compared with the normal population, *HLA-B*58:01* was associated with a higher risk of sCADR, both DRESS [odds ratio (OR) 85·36, 95% confidence interval (CI) 32·52–224·04] and SJS/TEN (OR 99·59, 95% CI 17·91–553·72). There was no statistically different risk between these two types of CADR.
Conclusions Portuguese patients with sCADR from allopurinol, both DRESS and SJS/TEN, have a high frequency of *HLA-B*58:01*, with an OR similar to European patients with SJS/TEN. This study also extends this association to DRESS in Europeans. The recommendation to genotype systematically before therapy is controversial, particularly when *HLA-B*58:01* prevalence in the normal population is low, as in Europe. However it could be an option for patients with other risks factors.

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What's already known about this topic?

- Allopurinol-induced Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) and DRESS in Han Chinese occur almost exclusively in *HLA-B*58:01*-positve individuals. A less strong association with *HLA-B*58:01* and allopurinol-induced SJS/TEN is observed in Europeans.

What does this study add?

- In a European population, *HLA-B*58:01* is also associated with DRESS, with a similar strength as for SJS/TEN.
- With a low prevalence of *HLA-B*58:01* in Europeans and a less strong association, systematic genotyping before allopurinol therapy remains controversial.

Allopurinol, widely used to lower blood uric acid levels and prevent or treat its complications, is associated with cutaneous adverse drug reactions (CADR) in 2–3% of exposed individuals. Severe CADR (sCADR), sometimes fatal, occur in up to 0·4%.[1,2] It is one of the most frequent causes of sCADR worldwide[3–7] and responsible for most cases of CADR requiring hospitalization in Portugal.[8]

Allopurinol causes several patterns of CADR due to delayed hypersensitivity, namely maculopapular exanthema (MPE), DRESS (drug reaction with eosinophilia and systemic symptoms), Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN)[4] and, less frequently, fixed drug eruption (FDE)[9] or acute generalized exanthematous pustulosis (AGEP).[7,10] Apart from skin resident or migrating cells (keratinocytes, dendritic cells, macrophages, natural killer T cells), drug-specific T cells (CD4+, CD8+, Th1, Th2, Th17) orchestrate the effector response and produce the final CADR phenotype.[11] Different T-cell subtypes are preferentially involved in each clinical pattern, but CD8+ granulysin-rich T cells that recognize antigens bound to human leucocyte antigen (HLA) class I molecules have a potent cytotoxic effect on keratinocytes and play an important role in the epidermal necrolysis in SJS/TEN,[12] and eventually also in epidermal aggression in MPE[13] and DRESS.[14]

At present, several *HLA* haplotypes are associated with a high risk for some CADR, particularly *HLA-B*57:01* for abacavir hypersensitivity syndrome,[15,16] and *HLA-B*15:02* for carbamazepine-induced SJS/TEN in Han Chinese.[17–19] This latter association is phenotype specific (only SJS/TEN) and does not extend to Europeans and Japanese where *HLA-A*31:01* is the main predisposing factor, both for DRESS and SJS/TEN induced by carbamazepine.[20] *HLA-B*58:01* has been associated with allopurinol-induced CADR, mostly in Han Chinese populations where almost all patients developing sCADR carry this allele.[21–24] This association is not as strong among other Asian and Australian patients; in Japan, only 36–40% are *HLA-B*58:01* positive.[6,25] In the European multicentre study published by Lonjou *et al.* concerning 31 patients with SJS/TEN, *HLA-B*58:01* was present in 19 (61%), but only in 15 out of 27 of European ancestry (55%).[26]

As there are few studies from Europe, the objective of our work was to evaluate whether this *HLA* allele also has a higher association with allopurinol-induced sCADR in our population and to calculate the relative risk of *HLA-B*58:01* carriers in developing sCADR from allopurinol.

Patients and methods

We conducted a retrospective and prospective study in patients with sCADR, including DRESS and SJS/TEN, imputed with high probability to allopurinol, who were admitted to the dermatology award of Coimbra University Hospital in the centre of Portugal. Patients hospitalized between 2005 and 2009, who responded voluntarily to our request in 2010 were tested retrospectively, whereas patients admitted between 2010 and 2012 were systematically tested.

Patients admitted with other diagnoses, who had been taking allopurinol for more than 4 months with no cutaneous adverse effects, were included as allopurinol-exposed and -tolerant controls. In addition, we used data from a control population of volunteers from the centre of Portugal who were typed for the Bone Marrow Donation Registry.

HLA typing was performed on a fresh sample of peripheral blood by reverse sequence-specific oligonucleotide–polymerase chain reaction using DNA extracted with Biorobot QiaSymphony and QiaSymphony DNA mini kits from Qiagen (Hilden, Germany). *HLA-B*58:01* was genotyped using OneLambda LABType® SSO-HR typing kits with Luminex® xMAP® technology confirmed by direct SBT Resolver™ kits from Conexio-Genomics (Freemantle, WA, Australia).

The association between *HLA-B*58:01* and allopurinol-induced sCADR was evaluated by comparing the group of individuals with sCADR with the allopurinol-exposed and -tolerant groups and the normal population. A similar comparison was performed separately for patients with DRESS and patients with SJS/TEN. Statistical analysis was performed using IBM SPSS statistics 20 (IBM, New York, NY, U.S.A.). We used the χ^2 test, considering $P < 0{\cdot}05$ as statistically significant, and odds ratios (OR) with 95% confidence intervals (CI).

The ethics committee of the Faculty of Medicine of Coimbra approved the study, as part of a more generalized study on CADR.

Patients and controls

We included 25 white patients, 11 male, 14 female, aged 29–93 years (mean 67·4) with sCADR imputed with high or very high probability to allopurinol, according to the French Pharmacovigilance criteria.[27]

Except one patient taking 100 mg, all were taking allopurinol 300 mg daily, prescribed for gouty arthritis in one case and asymptomatic hyperuricaemia in 21 – two with associated nephropathy. Information was not available in three cases (Table 1). CADR began within 3 days (in a case of involuntary reintroduction in a patient with TEN) up to 60 days in a case of DRESS (mean 23·4 days). Most patients were on multiple drugs, but allopurinol was the only one introduced *de novo* in the past 8 weeks. In one patient there was an accidental drug reintroduction with a MPE in the first exposure and TEN 3 years after that (patient 3) (Table 1).

In 19 patients, exfoliative erythroderma or longstanding generalized exanthema with facial oedema, fever, skin biopsies and laboratory tests, including abnormal liver or kidney function tests, eosinophilia, activated lymphocytes and human herpesvirus-6 viral reactivation, confirmed the diagnosis of DRESS, mostly as 'definite' or 'probable' cases according to the Regiscar criteria.[28] Severe hepatic failure occurred in two patients, one who also developed a severe deterioration of renal function, now on the waiting list for kidney transplantation. Generalized exanthema with atypical target and bullous lesions consistent with the diagnosis of SJS, overlap SJS/TEN

Table 1 Characteristics of the 25 patients who developed severe cutaneous adverse drug reactions (CADR) to allopurinol – demographic data, clinical aspects of CADR and human leucocyte antigen (HLA)

Patient no.	Sex	Age (years)	CADR type	Time interval (days)	Indications for allopurinol	Particular aspects	*HLA-A*	*HLA-B*	*HLA-DRB1*
1	M	69	TEN	15	Gout		*02*26	*44**58:01**	*07*13
2	F	76	SJS	15	n.a.		*02*34	*08*51	*04*07
3	F	78	TEN	3	AH	RI pos	*0*24	*35**58:01**	*04*12
4	F	76	SJS/TEN	12	AH		*01*30	*37**58:01**	*07*07
5	M	74	SJS	14	AH		n.a.	*07*44	n.a.
6	M	81	SJS/TEN	10	AH		n.a.	*B44**B58:01**	n.a.
7	F	75	DRESS	25	AH		*01*02	*44**58:01**	*07
8	M	47	DRESS	30	AH	PT neg	*01*34	*08	*03*07
9	F	76	DRESS	15	AH		*01*01	*14**58:01**	*07*13
10	M	76	DRESS	30	AH		*02*68	*50*51	*07
11	F	35	DRESS	16	AH	PT neg	*02*23	*50**58:01**	*03*07
12	F	81	DRESS	n.a.	AH		*23	**58:01**	*03
13	F	77	DRESS	21	AH	PT neg	*01*29	*44**58:01**	*07
14	F	75	DRESS	21	n.a.	PT neg	*11	**58:01**	*28
15	M	69	DRESS	60	AH		*31*34	*08*47	*03*12
16	F	73	DRESS	42	AH	PT neg	*03*03	*18*44	*07*13
17	F	68	DRESS	37	AH		*02*11	*35**58:01**	*12*13
18	M	46	DRESS	30	AH		*29*34	*08*51	*03*08
19	F	37	DRESS	21	AH + neph	Severe DRESS	*01 *01.	*08**58:01**	*03*04
20	M	93	DRESS	12	n.a.		*02*31	*40*44	*04
21	M	29	DRESS	21	AH + neph	Severe DRESS	*02	*35**58:01**	*11
22	F	68	DRESS	22	AH	100 mg/day	*02*34	*08*51	*03*07
23	M	77	DRESS	49	AH	PT neg	*25*68	*18**58:01**	*04*13
24	F	67	DRESS	21	AH	PT neg	*01*11	*13**58:01**	n.a.
25	M	63	DRESS	20	AH		*02*24	*35**58:01**	*07
Total average	11M/14F	67·4	6 SJS/TEN 19 DRESS	23·42				B*58:01 in 16	

DRESS, drug reaction with eosinophilia and systemic symptoms; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis; SJS/TEN, overlap SJS/TEN; AH, asymptomatic hyperuricaemia; neph, nephropathy; RI pos, positive accidental reintroduction; PT neg, negative patch tests; n.a., data not available. Bold face indicates presence of *HLA-B*58:01*.

and TEN defined, respectively, with < 10%, 10–30% and > 30% skin detachment,[29] was observed in six patients (Table 1).

In seven patients with DRESS patch testing was performed, according to the European Society for Contact Dermatitis guidelines,[30] with allopurinol at 1% and 10% petrolatum (pet.) (Chemotechnique Diagnostics, Malmö, Sweden), pure powder of allopurinol, supplied by the pharmaceutical industry, and its metabolite, oxypurinol (99·5% purity; Sigma Aldrich, St Louis, MO, U.S.A.) prepared locally at different concentrations and vehicles: allopurinol (1%, 10% and 20% pet.), oxypurinol (1% and 10% pet.) and both substances at 1% and 10% in ethanol and acetone.[31]

Additionally, we studied a group of six patients (three male/three female), aged 49–86 years (mean 70·3), hospitalized for an MPE induced by allopurinol, with minor systemic symptoms, who did not fulfil the criteria for DRESS.

The group of allopurinol-exposed and -tolerant controls consisted of 23 individuals, 16 male, seven female, aged 22–87 years (mean 62·0), with other dermatological diseases (erysipelas, venous leg ulcer, progressive systemic sclerosis, pemphigus erythematosus) who voluntarily agreed to collaborate in the study. They had been taking allopurinol 300 mg daily for more than 4 months, for gout (three) or asymptomatic hyperuricaemia (20), with no cutaneous adverse effects.

In addition, we used *HLA* prevalence data, based on the typing of a control population of 3200 individuals (65% female, 35% male; mean age 32 years) from the central region of Portugal, voluntarily typed for the Bone Marrow Donation Registry.

Results

The study included a group of 25 patients of European descent with a diagnosis of sCADR imputed with high probability to allopurinol, characterized phenotypically as DRESS (n = 19) or in the spectrum of SJS/TEN (n = 6), according to generally accepted criteria.[32] Patch testing with allopurinol and oxypurinol in different concentrations and vehicles was always negative, as referred to above.[31,33] Due to the severity of CADR no controlled drug rechallenge was performed.

The most frequently observed haplotype was *HLA-B*58*, always *HLA-B*58:01*, found in 16 patients (64%), 14 carrying one allele and two homozygous (Table 1). This haplotype was found in one of the 23 allopurinol-exposed and -tolerant con-

Table 2 Comparative frequencies of *HLA*-B*58:01 in patients with severe cutaneous adverse drug reactions (CADR) to allopurinol in allopurinol-exposed and -tolerant individuals and in the general population with calculation of odds ratio (OR) for the different types of CADR

	CADR from allopurinol		Allopurinol tolerant				Normal population			
	HLA-B*58:01/total	%	*HLA*-B*58:01/total	%	OR	95% CI	*HLA*-B*58:01/total	%	OR	95% CI
All severe CADR	16/25	64·0	01/23	4·3	39·11	(4·49–340·51)	63/3200	1·96	88·52	(37·69–207·92)
DRESS	12/19	63·2			37·71	(4·13–343·87)			85·36	(32·52–224·04)
SJS/TEN	4/6	66·7			44·00	(3·18–608·19)			99·59	(17·91–553·72)
MPE	1/6	16·6			n.s.				n.s.	

DRESS, drug reaction with eosinophilia and systemic symptoms; SJS/TEN, Stevens–Johnson syndrome or toxic epidermal necrolysis; MPE, maculopapular eruption; CI, confidence interval; n.s., not significant.

trols (4%), and in 63 of the 3200 individuals of the control population (1·96%). Using the χ^2 test the difference between the allopurinol-sCADR group and the allopurinol-tolerant group was statistically significant ($P = 5{\cdot}9 \times 10^{-4}$), particularly when comparing the sCADR group with the normal population. The presence of *HLA*-B*58:01 represents an OR 39·11 (95% CI 4·49–340·51) when compared with allopurinol-tolerant individuals, or 88·52 (95% CI 37·69–207·92), when using data from the normal population (Table 2).

HLA-B*58:01 was positive in 12 of 19 patients with DRESS (63%) and four of six with SJS/TEN (67%). When compared with the group of allopurinol-tolerant individuals this represents an OR 37·71 (95% CI 4·13–343·87) for DRESS and OR 44·0 (95% CI 3·18–608·19) for SJS/TEN. Considering the normal population it represents an OR 85·36 (95% CI 32·52–224·04) for DRESS and an OR 99·59 (95% CI 17·91–553·72), with no statistically significant difference between these two clinical types of CADR (Table 2).

HLA-B*58:01 was present in 11 of 14 female patients (79%) and in five of 11 male patients (46%). This sex difference was particularly evident in patients with DRESS, where nine of 11 women (82%) were positive, whereas only three out of eight men (38%) were *HLA*-B*58:01 carriers.

Only one among the six patients with a MPE had this allele, which is not statistically different from the allopurinol-tolerant group ($P = 0{\cdot}87$).

For other more frequent *HLA* alleles in patients with sCADR, *HLA-A*01* and *-A*02*, present in 32% and 45%, respectively, there was no significant difference from the normal population where they occur in 21·2% and 56·0%, respectively. For the B locus, *HLA-B*08* and *-B*51* found in 27% and 18%, respectively, of patients with sCADR, similar percentages occur in the normal population (12·8% and 20·9%); similarly for the more prevalent *DRB1*03*, *DRB1*04* and *DRB1*07*.

Discussion

Our study confirms that the strong association between *HLA*-B*58:01 and severe hypersensitivity CADR induced by allopurinol is also found in patients of European descent, with ORs between 39 and 88·5, respectively, when compared with allopurinol-tolerant individuals or the normal population. A similar OR for SJS/TEN has previously been observed in Europeans,[26] but this study also extends the risk to DRESS in patients with a European ancestry.

The association between *HLA*-B*58:01 and severe hypersensitivity reactions to allopurinol was first suggested in southern Chinese as early as 1989 by S.H. Chan and T. Tan (reported in ref. 34). This relationship has been more widely explored, particularly since 2005, when Hung *et al.*[35] published 51 cases of sCADR (21 SJS/TEN and 30 DRESS) induced by allopurinol in Han Chinese, all of them carrying this haplotype, whereas this allele was present in only 15% of allopurinol-tolerant individuals. More recent studies comparing patients with sCADR and allopurinol-tolerant individuals or the normal population, confirm this strong association in Han Chinese from China,[23] Thailand,[36] Hong Kong[24] and Korea,[22,37] and those living in Australia,[6] with an OR varying from 34·00 (in smaller samples) to 348·88 (mean 96·60; 95% CI 24·4–381·00, $P < 0{\cdot}001$) in SJS/TEN[21] and also in DRESS.

Such a strong association between allopurinol-induced sCADR and *HLA*-B*58:01 as found in the Chinese has only been observed in one report from Europe, from the Italian island of Sardinia, where all 18 SJS/TEN cases were positive.[7] In the European multicentre study on SJS/TEN only 15 out of 27 (55%) patients carry *HLA*-B*58:01, conferring an OR of 80·08 when compared with the normal population.[26] The present study with 25 patients with sCADRs found a very similar OR (88·52), even with a limited number of cases (six) of SJS/TEN (OR 99·59).

We found no previous report on the association between DRESS and *HLA*-B*58:01 in Europeans. In this study with a significant number of DRESS cases (19), we showed a similar high risk for this pattern of CADR (OR 85·36) as in the European multicentre study for SJS/TEN. Also, ORs were not significantly different for DRESS and SJS/TEN in this study. Therefore we may assume that *HLA*-B*58:01-positive patients of European descent have a similar high risk both for SJS/TEN and DRESS, but apparently lower than that in Han Chinese. This discrepant strength of association may be related to the different prevalence of this *HLA* allele in the normal population, which is high in Han Chinese (10–15%) and low (1–2%) in Europeans.[1,26]

Among patients with DRESS in this study, the percentage of *HLA*-B*58:01 carriers was much higher in female subjects

(81%) than in male subjects (38%). We found no previous published report on this sex difference, which may be just a spurious association.

The association of *HLA*-B*58:01 with milder CADR, like MPE, is less well studied. Recently, Cao *et al.* found that all 22 Han Chinese with MPE were *HLA*-B*58:01 positive.[23] Among 12 Australian patients none was *HLA*-B*58:01 positive[6] and we found only one positive case in six, with no statistical difference from the allopurinol-tolerant group. Such a low number of patients studied does not allow a definite conclusion.

The pathogenic role of *HLA*-B*58:01 underlying allopurinol hypersensitivity is not as well understood as for other drugs. Abacavir and carbamazepine or its metabolites bind the antigen-binding pocket of the HLA molecule, respectively, *HLA*-B*57:01 and *HLA*-B*15:02, which is needed for CD8+ T-cell recognition and activation[38–40] or for the cytotoxic effector response against keratinocytes.[19] How allopurinol or oxypurinol combine with *HLA*-B*58:01 is not known precisely, but preliminary *in vitro* studies confirm the importance of this relationship. It is easier to generate allopurinol-/oxypurinol-specific T-cell lines from *HLA*-B*58:01-positive individuals.[41] Also, *in vitro*-expanded CD8+ T lymphocytes from the peripheral blood of patients with CADR show strong cytotoxicity only against autologous antigen-presenting cells carrying *HLA*-B*58:01, a process that is inhibited by blocking HLA-I molecules.[42] Interestingly, only a few expanded T cells showed drug-specific cytotoxicity or T-cell proliferation, which may explain the difficulty in having positive lymphocyte stimulation or proliferation tests to confirm the diagnosis of allopurinol induced-CADR.[9,43] Moreover, the possibility of generating drug-specific T cells from *HLA*-B*58:01-negative individuals,[41] generally fits in with data from our study and the European study showing *HLA*-B*58:01 is a strong risk factor but not absolutely necessary for developing CADR. Also, *HLA*-B*58:01 is not invariably associated with CADR. In Han Chinese, 9·9–14·8% (mean 10·9%) of the 741 of allopurinol-tolerant controls also carry this HLA molecule.[21–23,35–37] As is the case for carbamazepine,[44] in addition to a predisposing HLA type, T cells with high-affinity T-cell receptor are needed to recognize and proliferate on exposure to allopurinol/oxypurinol. Otherwise, when affinity is less strong, either more drug/metabolite molecules or a state of preactivation of T cells is needed for a significant T-cell proliferation and a CADR to develop. This may explain, respectively, why patients with chronic renal insufficiency with impairment of oxypurinol excretion or those with concomitant viral infection are more prone to sCADR.[39]

With the present universal recommendation to pretest for *HLA*-B*57:01, hypersensitivity from abacavir has almost completely disappeared and genotyping for *HLA*-B*15:02 in Han Chinese before carbamazepine therapy has significantly reduced SJS/TEN induced by this drug in Taiwan.[45] Genotyping before allopurinol is a matter of debate,[1,6,21,22,46,47] although new techniques are being developed to lower the cost and accelerate the procedures.[46,47] As the negative predictive value is well below 100%, particularly in Europeans, pretesting will not eliminate all cases. Moreover, the high prevalence of *HLA*-B*58:01 in allopurinol-tolerant controls in populations with a high prevalence of this allele, might unnecessarily limit the use of this cheap drug, and replace it with other xanthine oxidase inhibitors (febuxostat) or other uric acid-lowering agents, which may be less efficacious, more expensive or with fewer long-term safety studies.[1,48,49] However, pregenotyping in populations with a high prevalence of *HLA*-B*58:01 may prevent a significant number of severe, even fatal cases. One may question the cost-effectiveness of its regular use in populations with a prevalence of *HLA*-B*58:01 below 2%, such as ours. It would be better limited to individuals with other risk factors for CADR, namely those with chronic renal insufficiency, those on multiple drugs, especially diuretics, or patients with Asian and Indian-Asian ancestry,[6] particularly those with relatives who developed a CADR from allopurinol.[50]

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F.3. Concluding remarks

As shown in the published study, our data confirms the strong association between HLA-B*58:01 and severe CADR induced by allopurinol in Portuguese patients. Moreover, for the first time, the present study with a significant number of DRESS cases (19), confirms that, like in Han Chinese, HLA-B*58:01 is a risk factor also for DRESS in patients with a European ancestry (Hung et al. 2005a),(Tassaneeyakul et al. 2009) (Jung et al. 2011),(Kang et al. 2011),(Cao et al. 2012),(Chiu et al. 2012). This had been shown only for SJS/TEN in European patients (Lonjou et al. 2008).

We found that HLA-B*58:01 represented a strong risk factor for DRESS (OR=85.36; 95% CI 32.52-224.04), a risk that is similar to SJS/TEN, both in our study (OR=99.59; 95% CI 17.91-553.72) and in the European multicentre study of SJS/TEN, when considering only patients with European ascent (OR=80.08; 95% CI 34-187) (Lonjou et al. 2008). This risk is, nevertheless, far from the report from the Italian island of Sardinia, where all 18 SJS/TEN cases were positive for this allele (Atzori et al. 2012), with a risk very similar to the one found in Han Chinese. The Italian authors do not comment on this association, namely on the prevalence of this allele in their normal population or any particular association with an Asian ascent in the population of the island.

The strength of the association between HLA-B*58:01 and severe CADR from allopurinol observed in our study (OR=88.52; 95% CI 37.69-207.92) is inferior to the one found in Han Chinese from Taiwan, China, Korea and Thailand. In their population, for SJS/TEN and very similarly also for DRESS, the risk expressed as Odds ratio (OR) varied from 34.00 (in smaller samples) to 348.88 (mean 96.60; 95% CI 24.4-381.00) up to 580.3 in the initial study (Hung et al. 2005a),(Tassaneeyakul et al. 2009),(Jung et al. 2011),(Kang et al. 2011),(Somkrua et al. 2011),(Cao et al. 2012),(Chiu et al. 2012),(Lee et al. 2012b),(Maekawa et al. 2012). This discrepant strength of association in relation to ethnicity may be related with the different prevalence of this HLA

allele in the normal population, which is high in Han Chinese (10-15%) and low in Portuguese and other Europeans (1-2%) (Hershfield et al. 2013),(Lonjou et al. 2008). Nevertheless, other explanations must certainly exist. Different metabolic equipment for metabolizing allopurinol among these populations, as shown for their different capacity to metabolize alcohol may be one contributing factor. Also, different environmental exposure to chemicals and pollutants that induce epigenetic modifications on our DNA or exposure to distinct pathogens that may favour the development of a different repertoire of immune receptors (TCR), may also contribute to this different risk. Additionally, as it has been shown that memory T cell-responses to some human herpes virus infections, or to vaccination, cross-react with endogenous peptides in individuals with certain HLA haplotypes and may favour drug recognition (Karlin and Phillips 2014), the difference in chronic and prevalent viral infections in distinct areas of the globe, may be another possible explanation for the different associated risk. Eventually, different single nucleotide polymorphisms (SNP) that may be in linkage disequilibrium with HLA-B*58:01 in certain populations, as shown in a Japanese population (Maekawa et al. 2012), may represent an additional factor contributing to the development of CADR from allopurinol.

In our published study, the association between milder allopurinol-induced maculopapular exanthema and HLA-B*58:01 was found only in one case in six, with no statistical difference from the allopurinol tolerant group. In further studies we observed this association in another patient in 2013, in a total of 2 HLA-B*58:01 positive patients out of 7 cases of maculopapular exanthema induced by allopurinol. The number is too low to take any conclusions. Moreover, the published reports from 2 different populations with allopurinol induced maculopapular exanthema are contradictory. Cao *et al.* found that all 22 Han Chinese with maculopapular exanthema were HLA-B*58:01 positive (Cao et al. 2012) but, among 12 Australian patients, none was an HLA-B*58:01 carrier (Lee et al. 2012b). Therefore, further studies are needed to know the real relation between this HLA genotype and milder CADR from allopurinol.

Interestingly, in our study, among DRESS patients the percentage of HLA-B*58:01 carriers was much higher in females (9/11 - 81.1%) than in males (3/8 - 37.5%). This difference was not apparent in SJS/TEN, where we observed 2 positive cases out of 3 in each sex. We found no explanation for this sex difference that has not been previously published. This may be just a spurious association, as we reported in the publication, but sex may have some other unexplained influence.

Our results, showing that HLA-B*58:01 is a significant risk factor for developing severe CADR from allopurinol but that this HLA genotype is not absolutely necessary for developing the reaction, are in perfect agreement with the more recent *in vitro* studies. They have shown that allopurinol/oxypurinol reactive T cells can also be developed upon stimulation of cells from HLA-B*58:01 negative and allopurinol naïve patients, although the affinity for oxypurinol binding is much stronger when using HLA-B*58:01 cells (Yun et al. 2013),(Yun et al. 2014).

At present, there is an important controversy on the possible advantages of pretesting for HLA-B*58:01 before using allopurinol, and there is an open debate on this matter (Somkrua et al. 2011),(Jung et al. 2011),(Kostenko et al. 2011),(Lee et al. 2012b),(Hershfield et al. 2013),(Kwok and Kwong 2013).

HLA-B*58:01 is not absolutely necessary for the development of the CADR, and a significant percentage of allopurinol tolerant individuals also carry HLA-B*58:01. It is the example of 1 of our 23 controls (4.3%) and 9.9-14.8% (mean 10.9%) of the 741 of allopurinol tolerant controls tested among Han Chinese (Hung et al. 2005a),(Tassaneeyakul et al. 2009),(Kang et al. 2011),(Somkrua et al. 2011),(Jung et al. 2011),(Cao et al. 2012).

The recommendation to use, in all HLA-B*58:01 positive individuals, alternative xanthine oxidase inhibitors, like febuxostat or other uric acid lowering agents (Hershfield et al. 2013),(Garcia-Valladares et al.

2011),(Richette 2012), will unnecessarily limit the use of allopurinol that would be tolerated by more than 10% of these individuals. Moreover, costs for drug replacement would be higher and safety assessment for these newer drugs is not completely established. The unnecessary replacement may be particularly significant in populations where the prevalence of HLA-B*58:01 is high. Moreover, genotyping for HLA-B*58:01 will not detect all cases. In our population about 36% of those who develop a severe CADR from allopurinol do not carry this allele.

Therefore, both the positive predictive factor and the negative predictive factor are far from the values observed for HLA-B*57:01 and abacavir (negative predictive value = 100% and positive predictive factor = 55%) (Phillips et al. 2011). Based on these values, a recommendation to genotype for this allele before initiating therapy was generated worldwide and it almost completely eliminated abacavir hypersensitivity. In the case of carbamazepine, systematic pre-genotyping for HLA-B*15:02, which carries a much higher increased risk (2500x) of SJS/DRESS from carbamazepine in Han Chinese (Hashizume 2012), was also a well accepted measure that very significantly reduced SJS/TEN induced by this drug in Taiwan (Wei et al. 2012b).

At present, the HLA genotyping technique is becoming more accessible, but it is not yet widely available and, although relatively low-price (<50 €) on an individual basis, it might become very expensive for systematic testing all potential users of allopurinol. New techniques to detect HLA-B*58:01 are being developed in order to allow quicker results and with less costs (Kostenko et al. 2011),(Kwok and Kwong 2013), but still far regular use.

Apart from the price and availability, in populations with very low prevalence of this HLA, like ours, it will be necessary to test more than 100 individuals to find 2 potential cases where CADR can be prevented, which might further question its cost-effectiveness. But at present, it has not been evaluated if the costs of systematic HLA-B*58:01 detection is, eventually, inferior to the sum of the costs of treating the severe CADR and its consequences.

We could not have access to the direct cost of hospitalization of these patients in our hospital and the health costs in the months after the CADR. When taken together, they may be even higher than the cost of genotyping 100 patients. Apart from the direct costs of hospitalization, which are certainly very high in severe TEN or DRESS, particularly if they have to include hepatic transplantation occasionally necessary in DRESS, it would be necessary to consider also the time of inactivity and loss of productive days, the life-impairment during and after such severe CADR, namely the frequent muco-cutaneous sequelae in SJS/TEN, including visual impairment and oesophageal synechia, or organ insufficiency or autoimmune diseases in DRESS. Moreover, the mortality rate in DRESS and TEN, which may vary between 5 and 90%, cannot be overlooked.

Considering all these aspects and the frequency and severity of the allopurinol-induced CADR, genotyping before initiating therapy may be justifiable, at least in individuals with risk factors for allopurinol-induced CADR. These are specially patients with chronic renal insufficiency, on multiple drugs, specially diuretics, patients with Asian and Indian-Asian ancestry (Lee et al. 2012b) and, particularly, those with relatives who developed a CADR from allopurinol (Lee et al. 2013c).

In our population, most patients with CADR were poly-medicated and, as usual practice, allopurinol was prescribed as a frequent add-on to other drugs in hypertension and dismetabolic syndromes with asymptomatic hyperuricemia. It was already noticed in a previous study from our group and in many international studies that most CADR occur in the context of asymptomatic hyperuricemia (Halevy et al. 2008),(Santiago et al. 2008),(Santiago et al. 2010). Therefore, a strong recommendation to limit the use of allopurinol to symptomatic cases would probably be more cost-effective to reduce CADR than pre-genotyping for HLA-B*58:01. A campaign with recommendations to limit the use of allopurinol, eventually associated with genotyping in susceptible groups, should be organized by official institutions. Dermatological publications, even in journals for general family physicians (Cabral and Gonçalo 2010), and the teaching of family physician trainees that observe

severe cases in our ward, which are within our reach at present, certainly have a very limited effect. A more active action is really needed to reduce the number of severe CADR from allopurinol.

G.
BASIC RESEARCH
In vitro effect of drugs on dendritic-like cells

G. BASIC RESEARCH - *IN VITRO* EFFECT OF DRUGS ON DENDRITIC-LIKE CELLS[4]

G.1. Introduction

Delayed CADR involve drug-specific effector T cells that recognize the drug in the context of HLA molecules and migrate to the skin where they exert their aggressive capacity (Gonçalo and Bruynzeel 2012),(Hausmann et al. 2012),(Roujeau 2005). To generate drug specific T cells in sufficient numbers to cause CADR, antigen presentation must be very efficient and able to overcome the natural tendency to create tolerance to chemicals that humans come in contact with. Therefore, antigen presentation must occur in a setting that is adequate for sensitization and generating the different types of drug specific memory and effector T cells. In drug eruptions, we do not know precisely which antigen presenting cells take part in this process, where sensitization occurs and how costimulatory signals enhance drug presentation to the immune system, but we presume that, like in other delayed hypersensitivity reactions, dendritic professional antigen presenting cells play this role.

During sensitization, apart from HLA-drug-TCR recognition, costimulatory signals from antigen presenting cells or neighbouring cells are usually necessary for sensitizing a naïve T cell. In CADR, external signals are usually considered to enhance drug sensitization. Underlying immuno-inflammatory

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[4] The results of this chapter concerning allopurinol have been presented 12nd Congress of the Portuguese Society of Dermatology, Troia, 19-21 October 2012 and at the 42nd ESDR meeting in Venice, 19-22 September 2012. They were published in the proceedings of this last meeting as:

Severe adverse drug reactions from allopurinol. *In vivo* and *in vitro* studies to understand pathophysiology. Margarida Gonçalo, I Coutinho, J Martins, B Neves, A Silva, R Nunes, A Martinho, T Cruz, C Lopes. *J Invest Dermatol* 2012: 132: S56

diseases, viral infections or concomitant drugs are mostly recognized as the "danger signals" that potentiate the formation of drug protein adducts to be presented to the immune system (Lavergne et al. 2009),(El-Ghaiesh et al. 2012), or modify the cytokine environment and increase adhesion and costimulatory molecules on immune cells, and, consequently, promote either antigen presentation or the effector T cell reaction (Hausmann et al. 2012).

In ACD, it has been recognized that most contact sensitizers have an inherent capacity to induce an innate immune inflammation, a xenoinflammation, that acts as an auto-adjuvant effect promoting their own presentation to the immune system (Martin 2012),(McFadden et al. 2013).

Contact sensitizers stimulate different membrane or cytoplasmic pattern recognition receptors (PRRs) in skin cells, both keratinocytes and DC, and use the same pathways as pathogen associated molecular patterns (PAMPs) shared by several microbes. For instance, metals like nickel, cobalt and palladium directly interact with the Toll-like receptor 4 (TLR4), the receptor for LPS from Gram negative bacteria and dimmerize the receptor inducing activation of MyD88 and further dependent signalling pathways (Schmidt et al. 2010),(Rachmawati et al. 2013).

Contact sensitizers may also exert their stimulation indirectly through damage-associated molecular patterns (DAMPs) liberated from the intercellular matrix and exposed cells (Ainscough et al. 2013). As an example, the strong contact sensitizer 1-fluoro-2,4-dinitrobenzene (DNFB), widely used in experimental settings, induces the production of reactive oxygen species (ROS), extracellular ATP and matrix degradation products, which are recognized as endogenous danger signals (DAMPs) by the membrane associated TLR4/TLR2 or by cytoplasmic NOD-like receptors (NLR) (Ainscough et al. 2013),(Martin et al. 2011). As a consequence of the recognition of these external "danger signals", there is activation of intracellular signalling pathways, such as p38 mitogen-activated protein kinase (p38 MAPK), and nuclear transcription factors, such as nuclear factor kappa B (NF-κB) and activator-protein-1 (AP-1) (Takanami-Ohnishi et al. 2002),(Matos et al. 2005a),(Neves et al. 2011).

Moreover, electrophilic skin sensitizers activate Keap1-Nrf2-ARE pathway (Kelch-like ECH-associated protein / Nuclear erythroid factor-related factor-2 / antioxidant response element) inducing the cellular detoxifying enzymes, e.g. NAD(P)H quinone oxireductase (NQO1) and hemeoxygenase 1 (HMOX-1). This is the usual cellular response to a stressful stimulus in order to eliminate harmful oxygen radicals and return to the normal intracellular redox state (Ade et al. 2009),(Natsch 2010),(Martin et al. 2011).

Additionally, through these or other pathways involving the purinergic receptor $P2X_7$ and ATP, there is activation of the NLRP3 inflammasome and caspase-1 (Martin et al. 2011),(Martins et al. 2012). Caspase-1 further cleaves the cytokines IL-1β, IL-18 and IL-33 and promotes their secretion to the extracellular space, where the cytokines represent the initial response to danger signals and contribute to the "awakening" of the adaptive immune system (Corsini et al. 2013) (Fig. B.11, Page 48).

Therefore, upon skin exposure to contact sensitizers there is abundant and varied cytokine/chemokine secretion from epidermal and dermal cells (IL-1β, TNF-α, IL-6, IL-10, IL-12, IL-18, IL-22, IL-23, CXCL1, CXCL2, IL-8/CXCL8, CXCL9, CXCL10, CCL2) that, together, create an ambience that favours antigen presentation (Martin et al. 2011),(Honda et al. 2013).

Both as a consequence of this cytokine/chemokine ambience and a direct effect of the sensitizer, DC initiate a maturation process. DC increase their expression of costimulatory molecules and chemokine receptors that contribute to DC migration towards the lymph node and facilitate DC communication with T cells (CD54, CD40, CD83, CD80/86, CXCR4 and CCR7) (Basketter and Maxwell 2007),(Neves et al. 2008).

This innate capacity of contact sensitizers to activate directly DC has been studied *in vivo*, by visualizing the change in morphology and phenotypic markers of DC and observing the migration of the activated DC carrying the antigens to the draining lymph nodes. Moreover, DC activation has been widely studied *in vitro,* using human DC differentiated from circulating $CD34^+$ cells and commercial DC lines obtained from humans (THP-1, MUTZ-3, U-

937) (Ashikaga et al. 2006),(Aeby et al. 2010),(Nukada et al. 2011) or mice (FSDC - fetal skin derived DC line) (Girolomoni et al. 1995),(Neves et al. 2013). Each of theses DC lines shows superiority over the others in their capacity to express the different specific markers of DC activation, but all these DC are activated in some way when exposed to most contact sensitizers (Neves et al. 2013).

THP-1 cells, due to their capacity to increase IL-8 production and upregulate the expression of CD54/ICAM-1 and CD86 on exposure to the sensitizing chemicals and due to their highly reproducible response, are probably the human dendritic-like cells most widely used to discriminate between contact sensitizers and irritants and, recently, also between photosensitizers and phototoxic chemicals (Hino et al. 2008),(Martínez et al. 2013). Based on their capacity to overexpress CD54 and CD86, an *in vitro* test has been developed, the human-cell line activation test (h-CLAT), which evaluates the skin sensitizing capacity of chemicals and, tentatively, also their potency as sensitizers. This test has shown a good inter and intra-laboratory reproducibility and a good correlation when compared with other methods, namely the local lymph node assay (LLNA) (Nukada et al. 2011),(Nukada et al. 2012),(Hirota et al. 2013).

In the many *in vitro* studies with contact sensitizers, occasionally, one or a few systemic drugs, namely penicillin G, streptomycin and clofibrate, have also been included in the long panels of possible sensitizing chemicals (Nukada et al. 2011),(Nukada et al. 2012),(Hirota et al. 2013),(Takenouchi et al. 2013). Very few studies have systematically addressed systemic drugs inducing CADR in these models and have evaluated their capacity of to induce a xenoinflammation similar to contact allergens.

G.2. Objective of the study

It was our main objective to understand if systemic drugs that cause CADR induce an innate immune response on DC that might promote their own presentation to the immune system, similarly to contact sensitizers.

We took advantage of the technology previously used by our group in the IBILI/FMUC (Institute for Biomedical Investigation of Light and Image of the Faculty of Medicine of the University of Coimbra) and CNC/UC (Centre for Neuroscience of the University of Coimbra) and the know-how accumulated in the study of DC maturation induced by contact sensitizers and several other external stimuli (Cruz et al. 1999),(Cruz et al. 2001a),(Cruz et al. 2001b).

In the last 12 years, our group has shown, in FSCD, and, more recently, also in THP-1, that many contact sensitizers activate intracellular signalling pathways and nuclear transcription factors, modify the intracellular oxidative status of the cells and change DC phenotypic markers, consistent with DC maturation (Cruz et al. 2002),(Cruz et al. 2004),(Cruz et al. 2005),(Matos et al. 2005b),(Matos et al. 2005a),(Vital et al. 2005),(Neves et al. 2008),(Francisco et al. 2010),(Martins et al. 2012).

In the present work, we studied the *in vitro* effect of several systemic drugs on different pathways involved in the activation of THP-1 cells, namely on intracellular signalling pathways (p38 MAPKinase) and genes coding for DC maturation markers (CD40, CD83), pro-inflammatory cytokine/chemokines (IL-8/CXCL8, IL-1β, IL12B or IL-12p40), CXCL-10, IL-4, IL-13, IL-5) and cell detoxifying enzymes (HMOX-1).

The following drugs and chemicals were selected for the study:

1. Allopurinol that is frequently responsible for severe delayed CADR in our hospital (Santiago et al. 2010),(Brinca et al. 2011) and worldwide (Halevy et al. 2008),(Mockenhaupt 2012);

2. Oxypurinol, the active metabolite of allopurinol, that is considered responsible for the T cell mediated CADR (Hamanaka et al. 1998),(Yun et al. 2012b);
3. Amoxicillin and ampicillin, two betalactam antibiotics, that most frequently cause CADR, particularly the less severe form of maculopapular exanthema;
4. Carbamazepine, an aromatic anticonvulsant, that is commonly associated with severe forms of CADR in our hospital, namely DRESS and SJS/TEN (Santiago et al. 2010),(Brinca et al. 2011); carbamazepine epoxide, one of the possible intermediates of carbamazepine metabolism, was also initially tested, but as there were no evident results, experiments did not proceed (data not shown);
5. Sodium valproate, an anticonvulsant, which seldom causes CADR and is indicated as a "safe" drug for individuals with delayed CADR from carbamazepine (Mockenhaupt 2012),(Santiago et al. 2010);
6. LPS, the bacterial lipopolysaccharide from Gram negative binds which activates TLR-4, is a potent DC maturation stimulus, that activates DC in many *in vitro* models (Cruz et al. 2001a),(Takahashi et al. 2011);
7. DNFB (dinitrofluorobenzene), a potent skin sensitizer that strongly activates DC in many *in vitro* models, including THP-1 cells (Cruz et al. 2002),(Nukada et al. 2012), and sensitizes most individuals, as long as enough skin exposure occurs (Friedmann 2007).

Results of *in vitro* studies obtained with the stimulation of systemic drugs were compared with the effects of the potent DC maturation stimulus, LPS, and the strong contact sensitizer, DNFB, chemicals that activate several pathways in THP-1 cells.

G.3. Material and methods

G.3.1. Material

LPS from *Escherichia coli* (serotype 026:B6) was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), phospho(P-) and total p38 MAPK antibodies from Cell Signalling Technology (Danvers, MA, USA), alkaline phosphatase-conjugated secondary antibodies from GE Healthcare (Chalfont St. Giles, UK), protease and phosphatase inhibitor cocktails from Roche (Mannheim, Germany), polyvinylidene difluoride (PVDF) membranes from Millipore Corporation (Bedford, MA, USA), TRIzol reagent from Invitrogen (Barcelona, Spain), RNA Storage Solution from Ambion (Foster City, CA, USA) and iScript Select cDNA Synthesis Kit from Bio-Rad (BioRad, Hercules, CA, USA).

All other drugs and reagents were purchased from Sigma-Aldrich Química, SA (Sintra, Portugal). Chemicals were at the highest possible purity (often > 98%).

Systemic drugs were mostly dissolved in the culture fluid, but for insoluble drugs, like carbamazepine and DNFB, the chemicals were previously dissolved in DMSO.

For testing allopurinol and oxypurinol, these chemicals were added to the culture medium that was heated to 55ºC (the temperature used to inactivate Fetal Bovine Serum). Then, a 10 M sodium hydroxide solution was added until dissolution. Afterwards, the solution was neutralized with a 10 M hydrochloric acid at the beginning and with a 1 M solution when near a neutral pH. Detailed characteristics of chemicals and solvents used are shown in Table G.1.

G.3.2. Cell culture

THP-1 human monocytic cell line (ATCC TIB-202, American Tissue Culture

Collection, Manassas, VA) is a monocytic human derived cell line obtained from the peripheral blood on a young male patient with acute monocytic leukaemia, with the following HLA haplotype: A2, A9, B5, DRw1, DRw2.

The cell line was cultured and maintained at a cell density between $0.1x10^6$ and $1x10^6$ cells/mL in RPMI 1640 supplemented with 10% of inactivated Fetal Bovine Serum (FBS), 25 mM glucose, 10 mM Hepes, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.05 mM of 2-mercaptoethanol. Cells were subcultured every 3–4 days and kept in culture for a maximum of 2 months.

G.3.3. Cell viability assay

We found no previous indications of which concentrations of most drugs under study should be used in *in vitro* experiments. Therefore, we tried to obtain the drug concentrations that allowed a viability of cultured cells around 70%, after the full length of exposure of THP-1 cells to the chemicals. We tested cell viability of THP-1 cells after 24h exposure to different concentrations of the drugs. After testing a 1 mM and a 10 mM drug solution, the concentrations were increased or decreased depending on the results obtained with these concentrations.

Cell viability was assessed by Alamar Blue assay (O'Brien et al. 2000). Briefly, $0.1x10^6$ cells/well in a 96 well plate in a final volume of 0.2mL/well were exposed to different drug concentrations, for 24 h. Three hours before ending exposure, resazurin solution was added to each well to obtain a final concentration of 50 μM. Absorbance of the medium was then read at 570 and 600 nm in a standard spectrophotometer (SLT, Austria).

Drug concentrations causing a 30% loss of viability (EC_{30} values) were determined in 3 to 6 independent experiments and used hereinafter: carbamazepine (0.3 mM), sodium valporate (25 mM), allopurinol (0.75 mM), oxypurinol (2.5 mM), amoxicillin (25 mM), ampicillin (50 mM), DNFB (7 μM)

and LPS (1 μg/mL) (Table G.1).

The maximum concentration of the solvent DMSO used (0.12%, for carbamazepine) had no effect on cell viability (data not shown).

Table G.1 - Characteristics of the chemicals, concentrations and solvents used in the experiments

Chemicals used	CAS number	Purity	EC30	Solvent
Carbamazepine (CBZ)	298-46-4	99,8% dry basis assay (meets USP testing specifications)	300 μM	DMSO
Valproic acid (VAL)	1069-66-5	≥98% (GC)	25 mM	Culture medium
Allopurinol (ALP)	315-30-0	≥99% (TLC)	0.75 mM	Culture medium[a,b]
Oxypurinol (OXP)	2465-59-0	≥98% (HPLC)	2.5 mM	Culture medium[a,b]
Amoxicillin (AMX)	26787-78-0	Analytical Standard (99,3% HPLC area)	25 mM	Culture medium[a]
Ampicillin sodium salt (AMP)	69-52-3	93.6% anhydrous basis assay	50 mM	Culture medium
1-fluoro-2,4-dinitrobenzene (DNFB)	70-34-8	≥99%	7 μM	DMSO
Lipo-polysaccharide (LPS)		gel-filtration chromatography	1 μg/mL[c]	Water

[a] Culture medium was heated at 55º C;

[b] Sodium hydroxide was added until complete dissolution and pH subsequently neutralized with chloridric acid;

[c] Concentration required to induced DC maturation

EC30: concentration required to induce 30% loss of viability (see Fig. G.2 and Material and methods section); USP: United States Pharmacopeia; GC: gas chromatography; TLC: thin layer chromatography; HPLC: High pressure liquid chromatography; DMSO: dimethyl sulfoxide

G.3.4. p38 MPAKinase analysis by Western blotting

Cells were plated in a 6 well plate at a density of $1x10^6$/mL and treated with the selected drugs (Table G.1), for 1 h. Cells were then lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with 1 mM DTT, and protease and phosphatase inhibitor cocktails, for 30 min in ice. The nuclei and the insoluble cell debris were removed by centrifugation (12,000*g* for 10 min, at 4ºC). Protein concentration was determined using the bicinchoninic acid method and the total cell lysates were denatured at 95ºC, for 5 min, in sample buffer (0.125 mM Tris pH 6.8; 2%, w/v SDS; 100 mM DTT; 10% glycerol and bromophenol blue).

For Western blot (WB) analysis, 30 μg of protein obtained after cell lysis were loaded and electrophoretically separated on a 12% (v/v) sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and transferred into a PVDF membrane. Membranes were blocked with 5% fat-free dry milk (w/v) in Tris-buffered saline containing 0.1% (v/v) and Tween 20 (TBS-T), for 1h, at room temperature. Blots were incubated overnight at 4ºC with the primary antibody against phosphorylated p38 MPAKinase (P-p38 MAPK) (1:1000). Membranes were extensively washed with TBS-T and incubated with freshly prepared alkaline phosphatase-conjugated anti-rabbit antibody (1:20,000) for 1h at room temperature. The immune complexes were detected by membrane exposure to the ECF reagent for 5 min, followed by scanning for blue excited fluorescence on the Typhoon imager (GE Healthcare). The generated signals were analysed using TotalLab TL 120 1D v2009 software. To test whether similar amounts of protein were loaded for each sample, the membranes were stripped using sodium hydroxide 0.2M and reprobed with antibodies to total p38 MAPK. Blots were again developed with alkaline phosphatase-conjugated secondary antibody and visualized by enhanced chemifluorescence.

G.3.5. Gene expression analysis by real-time RT-PCR

Cells plated in a 6 well plate ($0.75x10^6$/mL, 2mL/well) were treated with the selected drugs (Table G.1), for 24 h. Briefly, as previously described by Neves *et al.* (Neves et al. 2011), RNA was extracted with TRIzol reagent, concentration was measured by OD260 using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and samples were kept in RNA Storage Solution (Ambion, Foster City, CA, USA) at - 80°C until use.

cDNA was obtained by reverse-transcription of 1 μg of total RNA using the iScript Select cDNA Synthesis Kit. Real-time RT-PCR reactions were performed on a Bio-Rad MyCycler iQ5 as previously described (Neves et al. 2013). After amplification, a threshold was set for each gene and Ct values calculated for all samples. Gene expression changes were analysed using the built-in iQ5 Optical system software and results normalized using GAPDH as reference gene.

Primer sequences were designed using Beacon Designer software version 7.7 Premier Biosoft International (Palo Alto, CA, USA) and thoroughly tested. Forward (F) and reverse (R) primers used were as follows:

1. for *HMOX-1* - F: CCT GAG TTT CAA GTA TCC and R: AAC AAC AGA ACA CAA CAA;
2. for *IL-8/CXCL8* - F: CTT TCA GAG ACA GCA GAG and R: CTA AGT TCT TTA GCA CTC C;
3. for *CD40* - F: TGA TAG TGA ACA ACT GGA and R: CCA TAG GCA ATA TAC ATA CAT AA;
4. for *CD83* - F: ATT GAG TCA TTA TCC TTG CTA and R: GCT TCT TGG TAA CCT TCT T;
5. for *IL12B (IL-12p40)* - F: TGT CGT AGA ATT GGA TTG GTA TC and R: AAC CTC GCC TCC TTT GTG; and
6. for *CXCL10* - F: CCA GTC TCA GCA CCA TGA ATC and R: GCA GGT ACA GCG TAC AGT TC.

G.3.6. Data analysis

Real-time RT-PCR results represent the ratio of chemical-treated samples over untreated cells (control). A two-base logarithmic transformation was used to make observations symmetric and closer to a normal distribution. If x represents the normalized fold change of a gene in one sample, then the two-base logarithmic transformation ($log_2(x)$) is ln(x)/ln(2). Therefore, normalized fold changes of 2 and 0.5 correspond to mean log_2 values of 1 and -1, respectively.

Results are presented as mean ± SEM of the indicated number of experiments, and statistically compared with one-sample t-test or one-way ANOVA (when applicable) using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California, USA (www.graphpad.com). $P<0.05$ was considered significant.

G.4. Results

G.4.1. Cell cytotoxicity induced by systemic drugs

The cell viability aimed in most *in vitro* studies using systemic drugs or *in vitro* studies performed to evaluate the sensitizing capacity of contact sensitizers is between 70% and 75% (Nukada et al. 2011),(Sebastian et al. 2012). Therefore, using the Alamar Blue assay, drug concentrations causing near 30% cytotoxicity (EC_{30} values) were determined and used in further experiments in this study.

As previously shown for contact sensitizers, systemic drugs tested showed different cytotoxic potential on THP-1 cells, with the calculated EC_{30} values varying between 0.3 mM and 50 mM (Fig. G.2).

The EC_{30} values for allopurinol and carbamazepine were below 1 mM, respectively 0.3 and 0.75 mM. These two drugs, that cause most severe CADR, were the most toxics among all drugs tested. Nevertheless, their cytotoxicity is much lower than the strong sensitizer, DNFB (EC_{30} value 0.007 mM) (data not shown).

Allopurinol was more toxic than its metabolite, oxypurinol, whose EC_{30} value was 2.5 mM, whereas it was 0.75 mM for allopurinol.

Comparing the 2 anticonvulsants, sodium valproate, which seldom induces CADR, also induced much less cytotoxicity than carbamazepine.

In agreement with previous published studies using penicillin derivatives, ampicillin and amoxicillin induce relatively low cytotoxicity (Nukada et al. 2011),(Sebastian et al. 2012). Although these drugs frequently cause delayed CADR, these are usually the less severe forms.

There seems to be some correlation between the intensity of *in vitro* drug cytotoxicity and the frequency and, particularly, the severity of the CADR they cause.

By analogy with contact sensitizers, *in vitro* drug induced cytotoxicity may somehow be correlated with the drug sensitizing potential.

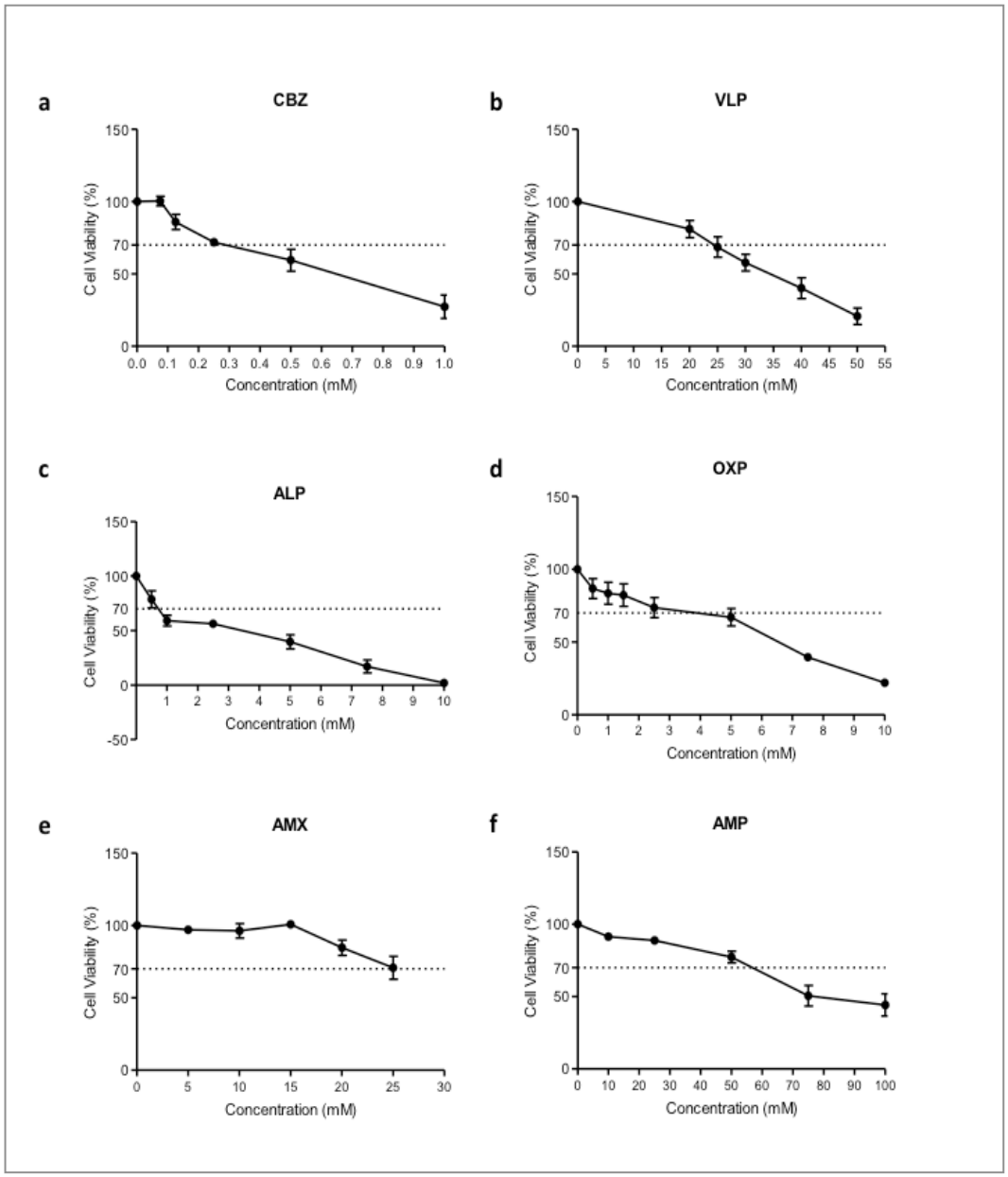

Figure G.2 – Determination of drug concentration that reduce 30% cell viability (EC_{30}). Cells in a 96 well plate ($0.1x10^6$/mL in a final volume of 0.2mL/well) were exposed to different concentrations of the drugs, for 24 h. Cell viability was assessed by Alamar Blue assay (50 μM) as described in *Material and methods* section. A drug concentration inducing 30% of cell death (EC_{30}), depicted by the point of the curves intersecting the dotted line corresponding to 70% of cell viability, was determined and used in subsequent experiments. The concentrations tested (mM) are shown in the graphics for each drug used: (**a**) Carbamazepine (CBZ); (**b**) Valproic acid (VLP); (**c**) Allopurinol (ALP); (**d**) Oxypurinol (OXP); (**e**) Amoxicillin (AMX); (**f**) Ampicillin (AMP). Data in graphs are means ± SEM of 3-6 independent experiments.

G.4.2. Hemeoxygenase–1 (*HMOX-1*) upregulation in response to systemic drugs and other exogenous stimuli

After 24 hours of cell stimulation there was an increase in the expression of the *HMOX-1* gene with most drugs, particularly sodium valproate, allopurinol and oxypurinol, suggesting the involvement of a toxicity pathway specifically activated by electrophilic cysteine-reactive compounds, namely by contact sensitizers (Ade et al. 2009),(Neves et al. 2013).

Both allopurinol and oxypurinol had a significant effect on *HMOX-1*, showing an increase in gene expression relatively to untreated cells (mean $\log_2$ values: 2.111 ± 0.309, $p< 0.05$ and 3.096 ± 0.575, $p<0.05$, respectively). These values are similar to those induced by the strong sensitizer DNFB (2.206 ± 0.529), but significantly inferior to LPS (5.997 ± 0.809) (Fig. G.3c, Table G.2).

Both anticonvulsants significantly upregulated the expression of the *HMOX-1* gene, although more significantly for sodium valproate (mean $\log_2$ values: 3.120 ± 0.615, $p<0.01$) than for carbamazepine (mean $\log_2$ values: 1.419 ± 0.528, $p<0.05$) (Fig. G.3c, Table G.2). Carbamazepine epoxide (1mM), tested to evaluate if this carbamazepine intermediate metabolite had a stronger effect, showed no modulation on the expression of *HMOX-1* or any of the other genes studied (data not shown).

Ampicillin was the only betalactam that significantly increased *HMOX-1* gene expression (1.842 ± 0.164, $p<0.01$) (Fig. G.3c, Table G.2).

G.4.3. Modulation of *IL-8/CXCL8* chemokine gene expression by drugs and other stimuli

The activation of the IL-8/CXCL8 pro-inflammatory chemokine gene, partially dependent on the Nrf2-ARE pathway, is known to play an important role in the skin sensitization process (Ainscough et al. 2013). Moreover, IL-8/CXCL8 production is often used as a good marker to evaluate the *in vitro* sensitizing

potency of chemicals, namely using THP-1 cells (Nukada et al. 2008),(Mitjans et al. 2010),(Takahashi et al. 2011).

IL-8/CXCL8 mRNA levels were consistently upregulated by allopurinol and oxypurinol (mean log_2 values: 3.219 ± 0.680, $p<0.05$ and 4.729 ± 0.508, $p<0.05$, respectively), similar to DNFB (mean log_2 values: 3.559 ± 0.116, $p<0.01$), but inferior to LPS (mean log_2 values: 10.630 ± 0.529, $p<0.0001$) (Fig. G.3f, Table G.2).

Ampicillin and amoxicillin also significantly upregulated the expression of IL-8/CXCL8 gene (respective mean log_2 values: 1.076 ± 0.2475, $p<0.05$ and 0.959 ± 0.154, $p<0.05$), although not as strongly as allopurinol and oxypurinol.

No significant effect was observed with the two anticonvulsants at the concentrations tested (Fig. G.3f, Table G.2).

IL-1β gene expression, also partially dependent on the Nrf2–cascade, was not modulated by ampicillin, amoxicillin and carbamazepine, although LPS induced a 20 fold-increase in this gene (data not shown).

G.4.4. Upregulation of genes coding for phenotypic markers of dendritic cell maturation (*CD40* and *CD83*)

Contact sensitizers are known to promote DC maturation, both *in vivo* and *in vitro*, expressed phenotypically by an upregulation of costimulatory molecules and chemokine receptors (Neves et al. 2008),(Kimber et al. 2013). Among the genes for DC maturation markers, that included *CD40, CD83, CD86* and *CCR7*, only *CD40* showed some significant modification induced by drugs.

As expected, LPS, a potent inducer of DC maturation, robustly upregulated *CD40* and *CD83* gene expression (mean log_2 values: 4.280 ± 0.363 and 5.334 ± 0.835), as well as *CD86* (data not shown).

Among drugs tested, sodium valproate showed a significant, although discrete

increase of *CD40* (mean log_2 values: 1.594±0.672, $p<0.05$). The increase induced by ampicillin (mean log_2 values: 0.999±0.226, $p<0.05$), although significant, was inferior to sodium valproate and contrasts with a no significant effect from the other amino penicillin derivative (Fig. G.3a, Table G.2).

No significant effect was observed with allopurinol or its metabolite at the concentrations tested.

Sodium valproate was the only drug that showed some increase in the expression of the *CD83* gene, although not significant (Fig. G.3b, Table G.2).

The gene for CD86 is one of the phenotypic DC maturation markers expressed at the surface of THP-1 cells. It is upregulated by many contact sensitizers and is an endpoint evaluated by flow-cytometer in the h-CLAT (Nukada et al. 2011),(Maxwell et al. 2011). In our experiments, *CD86* was strongly upregulated by LPS, but no significant effect was induced by carbamazepine, amoxicillin or ampicillin (data not shown). A similar result was observed for the gene for the chemokine receptor *CCR7* involved in DC migration to the lymph nodes (data not shown).

G.4.5. Involvement of cytokine/chemokine genes in the polarization of Th1/Th2 cell response

The cytokine IL-12, evaluated only by the gene coding for its p40 particle (*IL12B*), is important to drive T cell response into a predominant Th1 profile. CXCL10, formerly designated as IP-10, is upregulated in an IFN-γ rich ambience and is expressed in the skin exposed to a contact sensitizer, even in the absence of the inducing cytokine (Nakae 2003). Expression of these two genes, that are prone to a Th1 response, showed divergent results within the group of drugs studies.

As usually reported, LPS significantly upregulated both genes (mean log_2 values: 7.620 ± 0.820 and 10.650 ± 1.973, $p<0.01$, respectively for *IL12B* and

CXCL10). Sodium valproate was the only drug significantly increasing *IL12B* (mean $\log_2$ values: 3.119 ± 0.615, $p<0.01$; Fig. G.3d, Table G.2). In which concerns *CXCL10* only oxypurinol significantly upregulated this gene (mean $\log_2$ values: 1.290 ± 0.266, $p<0.05$; Fig. G.3e, Table G.2).

Both ampicillin and amoxicillin down-regulated *CXCL10* mRNA levels, although significantly only for the latter (mean $\log_2$ values: -0.779±0.031, $p<0.01$; Fig. G.3e, Table G.2).

Since penicillin derivatives reduced the expression of Th1 related cytokine genes in our study, we also evaluated the modulation of genes coding for cytokines that favour a Th2 response (IL-4, IL-5 and IL-13). Th2 cells are mainly involved in immediate reactions, which are frequently induced by penicillins (urticaria and anaphylaxis) and, both Th2 and Th1 cells, are involved in maculopapular exanthema induced by amoxicillin. In our experiments, the genes coding for the cytokines *IL-4, IL-5* and *IL-13* were barely expressed in THP-1 cells and their expression was not affected by aminopenicillins or any of the other drugs studied (data not shown).

Table G.2. – LPS, drugs and DNFB induced alterations on THP-1 gene expression. Results were determined by RT-PCR after stimulation of THP-1 cells for 24 h and are presented as the mean log_2 values ± SEM of the normalized fold expression against the control

	Stimulus							
Gene	**LPS**	**CBZ**	**VLP**	**ALP**	**OXP**	**AMX**	**AMP**	**DNFB**
HMOX-1	**5.997** ± 0.809 **+++** n=6	**1.419** ± 0.528 **+** n=6	**3.120** ± 0.615 **++** n=5	**2.111** ± 0.309 + n=3	**3.096** ± 0.575 + n=4	0.891 ± 0.222 n.s. n=3	1.842 ± 0.164 **++** n=3	**2.206** ± 0.529 **++** n=6
IL-8/ CXCL8	**10.63** ± 0.529 **+++** n=5	-0.087 ± 0.199 n.s. n=5	0.335 ± 0.673 n.s. n=4	**3.219** ± 0.680 **+** n=3	**4.729** ± 0.508 **+** n=3	**0.959** ± 0.154 **+** n=3	**1.076** ± 0.247 **+** n=3	**3.559** ± 0.116 **++** n=3
CD40	**4.280** ± 0.363 **+++** n=5	0.256 ± 0.153 n.s. n=5	**2.697** ± 0.219 **++** n=3	-0.016 ± 0.153 n.s. n=3	0.490 ± 0.292 n.s. n=3	0.708 ± 0.463 n.s. n=3	**0.999** ± 0.226 **+** n=4	Not tested
CD83	**5.334** ±0.834 **++** n=5	-0.242 ± 0.263 n.s. n=5	1.594 ± 0.672 n.s. n=4	-0.138 ± 0.463 n.s. n=3	0.142 ± 0.443 n.s. n=3	-0.126 ± 0.449 n.s. n=3	-0.222 ± 0.3995 n.s. n=4	Not tested
IP-10/ CXCL10	**10.65** ± 1.973 **++** n=6	0.921 ± 0.846 n.s. n=6	1.330 ± 1.101 n.s. n=5	0.455 ± 0.3743 n.s. n=3	**1.290** ± 0.266 **+** n=3	**-0.779** ± 0.031 **++** n=3	-1.053 ±0.269 n.s. n=3	Not tested
IL12B	**7.620** ± 0.820 **+++** n=6	0.586 ± 0.485 n.s. n=6	**3.119** ± 0.615 **++** n=5	0.165 ± 0.289 n.s. n=3	-0.932 ± 0.390 n.s. n=3	-1.127 ± 0.420 n.s. n=3	1.002 ± 0.849 n.s. n=4	Not tested

Statistical analysis - One-sample t-test comparing to control (zero): n.s. – not significant; + - $P<0.05$; ++ - $P<0.01$; +++ - $P<0.001$.

n – number of independent experiments, CBZ – Carbamazepine, VLP – Valproic acid, ALP – Allopurinol, OXP – Oxypurinol, AMX – Amoxicillin, AMP – Ampicillin

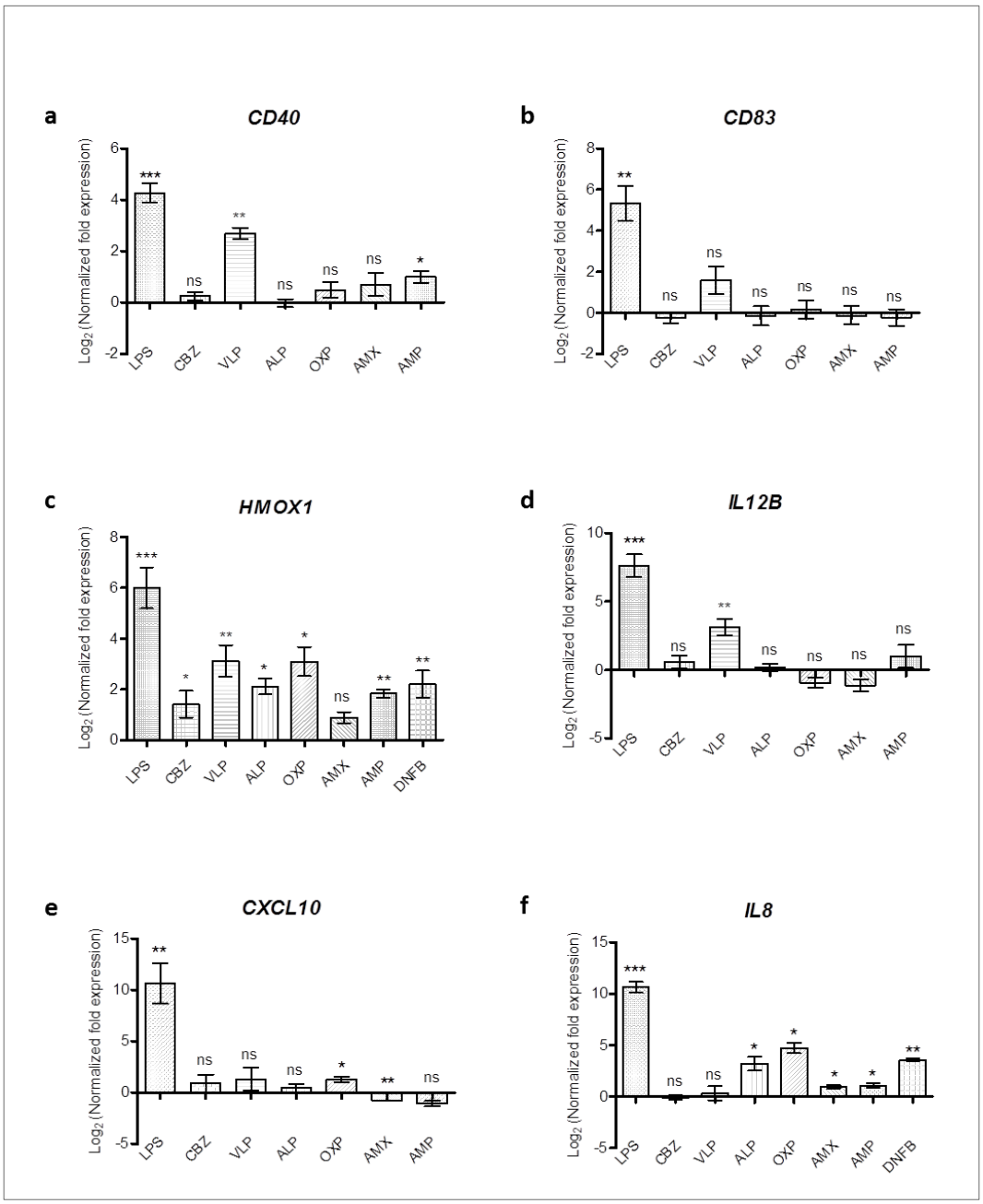

Figure G.3 – Modulation of gene expression induced by systemic drugs. Cells were treated for 24h with LPS (1 µg/mL), CBZ, VLP, ALP, OXP, AMX, AMP **(a-f)** and DNFB (in **c** and **f**) at EC_{30} concentrations. Gene expression of *CD40* (**a**), *CD83* (**b**), *HMOX-1* (**c**), *IL12B* (**d**), *CXCL10* (**e**) and *IL-8/CXCL8* (**f**) was analysed by real-time RT-PCR. Data correspond to mean log_2 values ± SEM of the normalized fold expression relatively to control cells determined on 3-6 independent experiments. Statistical analysis: One-sample t-test: ns – non significant; *P<0.05; **P<0.01 and ***P<0.001, compared to control (zero).

G.4.6. Concomitant stimulation of THP-1 cells by ampicillin and oxypurinol

Clinical data suggest that concomitant exposure to allopurinol and betalactam antibiotics may increase the probability of developing a CADR (Boston Collaborative Drug Surveillance Program 1972),(Ben Fredj et al. 2010). Therefore, we studied the effect of exposing THP-1 cells to a mixture of ampicillin and oxypurinol. We chose ampicillin and oxypurinol that had an effect superior to their analogues, respectively amoxicillin and allopurinol, when tested separately. We studied the modulation of the genes that had previously shown to be activated more intensely by these compounds in THP-1 cells, namely *IL-8/CXCL8* and *HMOX-1*.

Exposing cells for 24h to the mixture of ampicillin and oxypurinol, at the same concentrations previously used in individual experiments, showed no additive cytotoxicity (Fig. G.4), therefore the same drug concentrations were used in the mixture and in individual experiments.

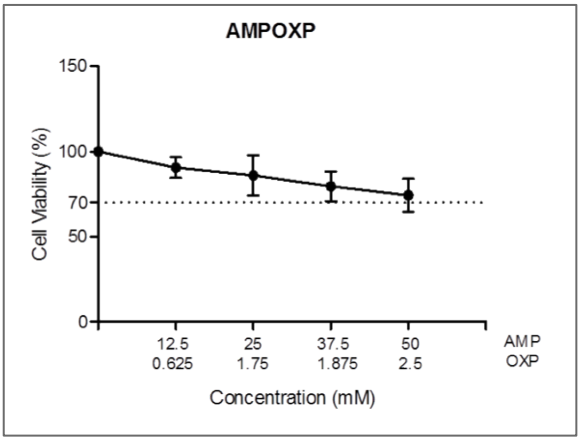

Figure G.4 – Determination of drug concentration of the mixture of oxypurinol and ampicillin that reduce 30% cell viability (EC_{30}). Like in the study with single drugs, THP-1 cells in a 96 well plate ($0.1x10^6$/mL in a final volume of 0.2mL/well) were exposed to different concentrations of the two drugs, for 24 h and cell viability was assessed by Alamar Blue assay, as described in *Material and methods* section. The mixture of AMP at 50mM and OXP at 2.5mM (AMPOXP), the concentration that reduced cell viability around 30% (EC_{30}) in isolated experiments, induced an identical loss of cell viability and was therefore used in subsequent experiments. Data represent means ± SEM of 4 independent experiments.

The combination of the two drugs (AmpOxp, Fig. G.5 g-h), in 4 unrelated experiments, lead to an upregulation of both *HMOX-1* and *IL-8/CXCL8* gene expression (mean log_2 value: 2.303 ± 0.542 and 1.989 ± 0.555, respectively). Nevertheless, this effect was inferior to the one observed in experiments using oxypurinol alone (mean log_2 value: 3.096 ± 0.575 for *HMOX-1* and 4.729 ± 0.508 for *IL-8/CXCL8*) and only slightly superior to ampicillin alone. These results suggest a possible antagonism rather than an additive effect.

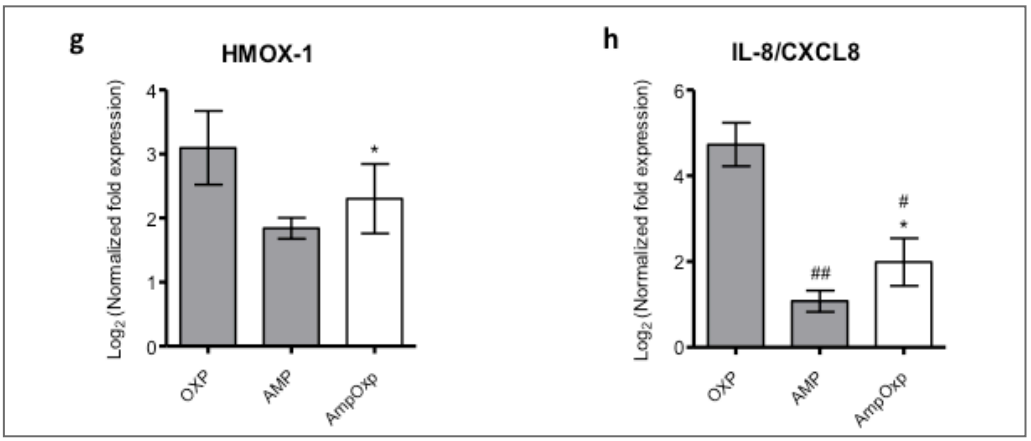

Figure G.5 –Modulation of *HMOX-1* and *IL-8/CXCL8* gene expression induced by the cumulative exposure to oxypurinol and ampicillin. Cells were treated for 24h with a mixture of OXP and AMP at EC_{30} concentrations. Gene expression of *HMOX-1* (**g**) and *IL-8/CXCL8* (**h**) was analysed by real-time RT-PCR. The cumulative effect of ampicillin and oxypurinol (AmpOxp) on *HMOX-1* (**g**) and *IL-8/CXCL8* (**h**) was compared with each drug alone (grey bars). Grey bars correspond to data depicted in Fig G.3**c** and Fig. G.3**f**, respectively. Data correspond to mean log_2 values ± SEM of the normalized fold expression relatively to control cells determined on 4 independent experiments. Statistical analysis: One-way ANOVA with Tukey's Multiple comparison test: $^{\#}P<0.05$ and $^{\#\#}P<0.01$, compared to OXP. $^{*}P<0.05$; $^{**}P<0.01$, compared to control (zero).

G.4.7. Activation of p38 MAPKinase by drugs

Danger signals and contact sensitizers activate several intracellular signalling pathways that control DC maturation and prepare these cells for antigen presentation. Our group and other investigators have shown that skin sensitizers activate MAPKinases, particularly the p38 MAPKinase, by increasing its phosphorylated form (P-p38MAPKinase). Moreover, this intracellular signalling pathway has been shown to be involved in the expression of DC maturation markers, namely CD40 (Arrighi et al. 2001),(Matos et al. 2005a),(Matos et al. 2005b),(Mitjans et al. 2010),(Neves et al. 2013).

Therefore, similarly to contact sensitizers, we exposed THP-1 cells to the chemicals for 1 hour and evaluated the effect of the different drugs on the activation of p38 MAPK, by assessing P-p38 MAPK by Western Blotting.

In five unrelated experiments we observed that all the drugs slightly induced the activation of p38 MAPK, similarly to DNFB (6.105 ± 2.998) and LPS (data not shown) (Fig. G.4).

This increase was statistically significant only for oxypurinol (1.829 ± 0.298; $p<0.05$), which contrasts with a non-significant activation induced by allopurinol (1.434 ± 0.2342) (Table G.3).

Activation of p38 MAPKinase observed with the anticonvulsants (1.653 ± 0.267 and 1.959 ± 0.507, respectively for carbamazepine and sodium valproate) as well as with the betalactams (2.196 ± 0.458 and 2.405 ± 0.838, respectively for amoxicillin and ampicillin), was not significant ($p> 0.05$).

Table G.3. – Activation of p38 MAPKinase by systemic drugs and the contact sensitizer DNFB. After stimulation of THP-1 cells for 1 h with the stimulants, results were determined by WB and presented as the increase of P-p38 MAPKinase in relation to control, in 5 independent experiments

	CBZ	VLP	ALP	OXP	AMX	AMP	DNFB
Mean	1,653	1,959	1,434	1,829	2,196	2,405	6,105
SEM	0,2674	0,5068	0,2342	0,2498	0,4581	0,8376	2,998
P value	0,0710	0,1314	0,1375	0,0294*	0,0594	0,1687	0,1638

*- significant result (P<0.05); CBZ – Carbamazepine, VLP – Valproic acid, ALP – Allopurinol, OXP – Oxypurinol, AMX – Amoxicillin, AMP – Ampicillin, DNFB – dinitrofluorobenzene.

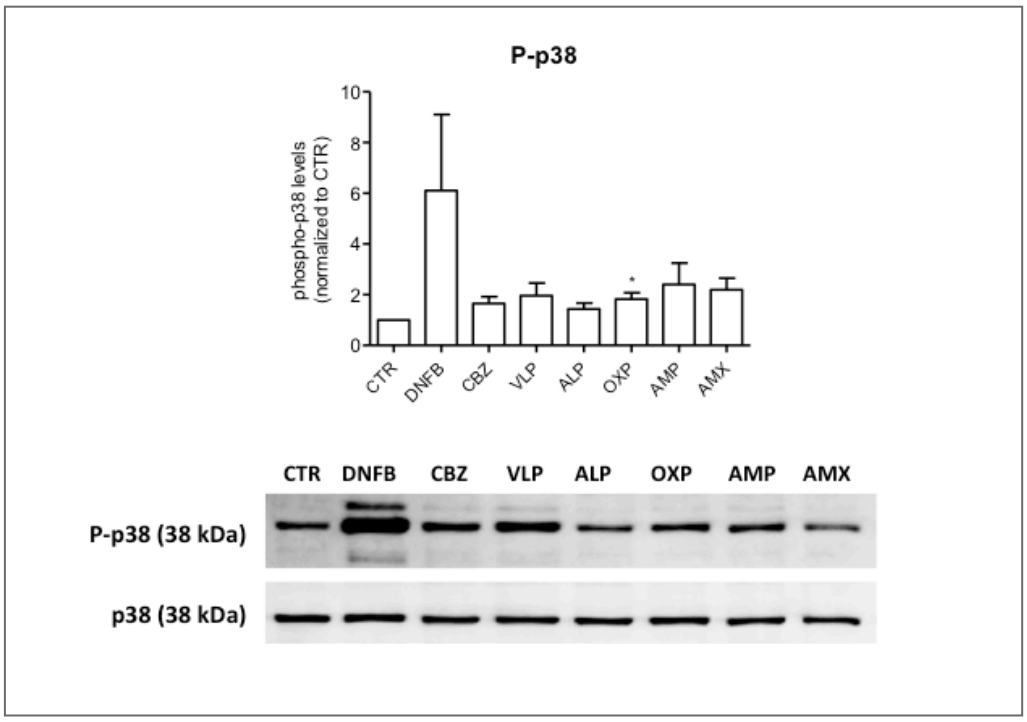

Figure G.6 – p38 MAPK activation by systemic drugs, compared to DNFB. Cells were plated in a 6 well plate (2x10^6/mL) and exposed to DNFB, CBZ, VLP, ALP, OXP, AMP and AMX at EC_{30} concentrations, for 1h. p38 MAPK phosphorylation levels (P-p38/p38 total levels) were analysed by Western blotting. Data in the graph are the mean ± SEM of 5 independent experiments, normalized to control (CTR). The image shows a representative blot of the data depicted in the graph. Statistical analysis – One-sample t-test; *P<0.05, compared to CTR.

G.5. Discussion

Similarly to contact sensitizers, that were shown to activate dendritic-like cells, including THP-1 cells (Nukada et al. 2011),(Kimber et al. 2013), we showed that several systemic drugs, that induce delayed CADR, also activate different, but relevant genes in THP-1 cells and use intracellular signalling pathways that are usually involved in DC maturation and activation.

Nevertheless, both the intensity of the response and the genes or signalling pathways activated were dependent on the drug, as is usually observed with different contact sensitizers (Neves et al. 2013),(Richter et al. 2013). Moreover, like contact sensitizers, the potency of drug-induced DC stimulation, as well as its cytotoxicity, varies with the drug, which in our model seems to be related with their capacity to sensitize or induce severe CADR.

G.5.1. The effect of systemic drugs on dendritic-like cells and its relevance for antigen presentation

Few studies have addressed the innate immune response during sensitization to systemic drugs. By analogy with contact sensitizers and ACD, dendritic cell maturation may represent a significant innate cellular response to the presence of systemic drugs and, consequently, it may be relevant to the pathophysiology of delayed immune mediated CADR.

In ACD, dendritic cell activation upon skin exposure to contact sensitizers is considered a key event in the physiopathology of ACD. Contact sensitizers directly enhance cytokine/chemokine secretion and maturation of skin DC, which promotes DC migration towards the regional lymph nodes and presentation of haptenized peptides to naïve T cells (Martin 2012). In CADR, cells that present drug-sensitizing epitopes to the immune system have not been clearly identified. *In vitro*, blood derived dendritic-like cells from patients

with delayed CADR can present drugs to T cells, as shown for sulfamethoxazole, carbamazepine and amoxicillin (Poszeczynska-Guigné et al. 2005),(Rodriguez-Pena et al. 2006),(Sanderson et al. 2007),(Park et al. 2007). However, other antigen presenting cells, namely skin DC, may also have an important contribution in the drug sensitization phase of CADR.

Systemic drugs reach the skin and use the metabolizing capacity of keratinocytes (Merk 2009). Therefore, they can also use the immune efficient skin DC to present drug-derived reactive epitopes to the immune system. This might explain the frequent skin involvement in delayed immune ADR and the reproducibility of the skin reaction when the drug is patch tested (Barbaud 2009),(Gonçalo and Bruynzeel 2011). Therefore, it is plausible that, in DC models, systemic drugs can elicit effects similar to contact sensitizers, as we observed in the several genes in THP-1 cells.

Penicillins and clofibrate, included among the contact sensitizers studied in the h-CLAT model, also induced an upregulation of the adhesion molecule CD54, but not the costimulatory molecule CD86, as evaluated by flow cytometer in THP-1 cells (Nukada et al. 2011),(Takenouchi et al. 2013).

In the present work, we did not study the final phenotypic changes in the THP-1 cells, as in the h-CLAT (Ashikaga et al. 2006). Nevertheless, we observed the activation of relevant signalling pathways (p38 MAPKinase) and genes coding for DC maturation markers, namely CD40. We did not evaluate gene expression for CD54, but similarly to the h-CLAT, we observed no upregulation in the expression of CD86 gene by penicillins, or any other drug.

The molecule CD40 is particularly expressed in mature DC and is involved in the cross talk between the DC and T cell during antigen presentation. In our study, gene transcription for CD40 was upregulated significantly only by sodium valproate and ampicillin. In other studies, CD40 and other DC maturation markers (CD86 and HLA-DR) were also upregulated by sulfamethoxazole and amoxicillin in moDC (Sanderson et al. 2007),(Rodriguez-Pena et al. 2006), but CD86 and HLA-DR upregulation occurred only in cells from patients with amoxicillin induced-maculopapular exanthema (Rodriguez-

Pena et al. 2006).

The different behaviour of moDC from sensitized versus non-sensitized patients, upon exposure to the drugs, may result from contamination of moDC with drug specific T cells in the sensitized patient. But, these differences might also correlate with distinct individual susceptibility factors for initiating CADR. Differences in the capacity of the moDC to metabolize the drug, may dictate distinct sensitizing epitopes, and distinct responses of the moDC in which concerns the innate recognition of drugs as "danger signals", may interfere at the initial phase of drug presentation to the immune system, before specific T cell recognition.

HLA molecules, that influence T cell recognition of drugs, were, until now, the strongest identified genetic risk factor for delayed CADR. Due to the particular drug binding with the some HLA molecules, we might expect that drug binding may also have some influence on the cellular response, but this may not be significant in the initial response. In our model with THP-1 cells, which express HLA-B*15 (Battle et al. 2013), allopurinol was particularly active although this drug is particularly recognized by the HLA-B*58:01 molecule (Yun et al. 2012b), which is also an important risk factor for severe CADR (Chiu et al. 2012),(Hung et al. 2005a) (Lonjou et al. 2008).

G.5.2. Systemic drugs as a cause of stress and activation of ARE-dependent genes

One of the strongest and more consistent responses obtained with all drugs was the upregulation of the gene coding the cellular detoxifying enzyme, *HMOX-1*. As this enzyme is involved in the resolution of oxidative and electrophilic stresses (Ade et al. 2009), we may assume that all the drugs studied caused a significant stress to THP-1 cells and represented "danger signals" that motivated different cellular responses, including *HMOX-1* and *IL-8/CXCL8* gene expression or, in extreme cases, cell cytotoxicity, as shown mainly for

allopurinol and oxypurinol.

As shown for contact sensitizers, drugs may behave as electrophilic chemicals and bind the cysteine-rich domain of Keap-1/Nrf-2 complex pathway, considered a cytosolic sensor for electrophilic chemicals. Upon binding the Keap-1 unity, Nrf2 is liberated and translocate into the nucleus. Here, it binds the antioxidant response elements (ARE) and promotes the transcription of detoxifying enzymes, including HMOX-1 (Migdal et al. 2013) and the IL-8/CXCL8 chemokine, that may be also depend on this pathway (Ainscough et al. 2013) (Fig. B.11, Page 48).

Except for penicillins, we found no previous studies on the effect of systemic drugs on the ARE dependent pathway. Penicillins have revealed divergent results on this pathway and on the related DC activation markers, with results depending on the DC-model and on the penicillin derivative used, benzylpenicillin (penicillin G), phenoxymethylpenicillin (penicillin V) or an aminopenicillin (Rodriguez-Pena et al. 2006),(Sebastian et al. 2012).

The divergent results may be due to the fact that, during the formation of adducts with proteins, penicillins bind mainly lysine residues in peptides, but they can also bind cysteine (Weltzien and Padovan 1998). Chemicals binding lysine are considered unable to activate the Nrf2-ARE pathway, particularly in THP-1 cells. These chemicals are mostly involved in Th2-related immediate reactions, like urticaria and angioedema, frequently observed with penicillins. The Nrf2-ARE pathway is activated mainly by compounds binding cysteine residues, namely contact sensitizers (Migdal et al. 2013), that induce mostly Th1 reactions. As aminopenicillins also bind cysteine residues in proteins (Weltzien and Padovan 1998), they might be expected to stimulate also the Nrf2-ARE pathway and, therefore, cause delayed immune mediated CADR with a predominant Th1 involvement, like maculopapular exanthema.

In agreement with the lysine-binding hypothesis, Megherbi *et al.* (2009) showed no activation of the Keap-1-Nrf2-ARE pathway by penicillins, in THP-1. On the opposite, and consistent with the cysteine binding properties of aminopenicillins, we observed that ampicillin significantly upregulated

HMOX-1 gene. Moreover, using also THP-1 cells, Sebastian et al. (2012) showed that penicillins, particularly penicillin V, upregulated another detoxifying enzyme, the NQO1, whose activation is also dependent on the Keap-1-Nrf2-ARE pathway. Additionally, on blood derived moDC, the detoxifying enzymes, along with IL-8/CXCL8, were highly upregulated by penicillin G and V, but not so intensely by ampicillin (Sebastian et al. 2012). Therefore, by their capacity to bind either lysine or cysteine, penicillins can activate different signalling pathways in DC, which may be relevant for the different types of CADR they can induce.

G.5.3. Systemic drugs in the activation p38 MAPKinase and other signalling pathways

Contact sensitizers frequently activate p38 MAPK (Arrighi et al. 2001),(Mitjans et al. 2010),(Neves et al. 2011). This intracellular signalling pathway was also activated by all the drugs tested in our model, although significantly only by oxypurinol.

Oxypurinol was, simultaneously, the most potent drug in the activation/phosphorylation of p38 MAPK and in the upregulation of the *IL-8/CXCL8* and *HMOX-1* genes. As IL-8/CXCL8 secretion in THP-1 stimulated by contact sensitizers seems to be related with p38 MAPK activation (Nukada et al. 2008),(Mitjans et al. 2010),(Takahashi et al. 2011), we may understand the potent effect of oxypurinol on *IL-8/CXCL8* gene expression.

Activation of p38 MAPK is highly involved in DC maturation and expression of its phenotypic markers (Arrighi et al. 2001),(Matos et al. 2005a),(Matos et al. 2005b),(Mitjans et al. 2010),(Neves et al. 2013). As, apart from oxypurinol, the effect of other drugs on p38 MAPK activation was rather low, we may understand that there was not a very significant upregulation of genes coding for DC maturation markers, with only a slight increase of *CD40* and *CD83* by ampicillin and sodium valproate.

The pathway leading to p38 MAPK activation by drugs has not been studied. In ACD, p38 MAPK is activated via TLR-4 and MyD88, as in the case of nickel, palladium and cobalt (Rachmawati et al. 2013), or via the Nrf2-Keap-1 pathway, particularly by contact sensitizers that induce the formation of ROS or hyaluronic acid fragments (Fig. B.11, Page 48). Contact sensitizers, that use the purinergic receptor $P2X_7$ and ATP, can directly activate the NLRP3 inflammasome and caspase 1 to cleave cytoplasmic pro-IL-1β and pro-IL-18, escaping the p38 MAPK pathway (Martin et al. 2011). Nevertheless, as shown by our group in THP-1 cells, other purinergic receptors (P2Y2 and P2Y11) activated by DNFB can also activate p38 MAPK (Martins et al. 2012).

In our study, other drugs also activated relevant genes without significantly activating p38 MAPK. Therefore, we may assume that other intracellular signalling pathways may be involved in DC stimulation by drugs. *IL-8/CXCL8* and *HMOX-1* genes, activated by most drugs, are also dependent on the ARE-pathway (Ainscough et al. 2013), which is usually activated by cysteine binding chemicals, as shown to occur with aminopenicillins, or in the presence of ROS.

Allopurinol inhibits xanthine oxidase, an enzyme that is an important generator of ROS. In this way, we might expect that allopurinol decreases the activation of ARE-dependent genes by reducing ROS as a "danger signal". Indeed, allopurinol reduced IL-18 production in keratinocytes stimulated by the contact allergens DNFB and p-phenylenediamine, presumably by inhibiting mitochondrial superoxide formation induced by the sensitizers (Corsini et al. 2013). On the other hand, both allopurinol and oxypurinol are also a substrate for xanthine oxidase and, in this way, may generate ROS. This might explain the increase in the ARE-dependent *HMOX-1* and *IL-8/CXCL8* gene expression, observed in our study.

Nevertheless, allopurinol and the other systemic drugs can use alternative pathways, independent from ROS and p38 MAPK, to activate THP-1 cells.

G.5.4. IL-8/CXCL8 as a "danger signal" or an enhancer of acute generalized exanthematous pustulosis

The chemokine IL-8/CXCL8, that represents an innate response of keratinocytes and DC to the presence of the contact sensitizer, is involved in the initial phases ACD (Sebastiani et al. 2002),(Frankart et al. 2012),(Mattii et al. 2013). Most studies confirm DC production of IL-8/CXCL8 induced by contact sensitizers, particularly *in vitro,* in blood derived moDC and in several dendritic-like cells, like THP-1 (Nukada et al. 2008),(Mitjans et al. 2010),(Takahashi et al. 2011),(Rachmawati et al. 2013). At present, IL-8/CXCL8 production is one of the major endpoints used to evaluate the contact sensitizing potential of chemicals (Mitjans et al. 2010)(Neves et al. 2013).

In this study, we demonstrated a very significant upregulation of *IL-8/CXCL8* gene expression induced by allopurinol/oxypurinol and by the aminopenicillins, similarly to contact sensitizers. Our results are somehow corroborated by a recent work showing that ampicillin, and other penicillins increased *IL-8/CXCL8* gene expression in moDC, although not in THP-1 cells (Sebastian et al. 2012).

Therefore, IL-8/CXCL8 may be an additional relevant signal in the innate response of cutaneous cells and, eventually, also other cells of our body, to the presence of systemic drugs.

IL-8/CXCL8, a potent neutrophil chemoattractant, is involved in delayed CADR, particularly in AGEP, characterized by neutrophilia and recruitment of neutrophils into the skin (Speeckaert et al. 2010). In AGEP, IL-8/CXCL8 is considered to be produced mainly by keratinocytes stimulated by Th17 cells (Kabashima et al. 2011),(Frankart et al. 2012), but DC and T cells can also produce this cytokine. In our dendritic-like cell model, both allopurinol and aminopenicillins significantly upregulate *IL-8/CXCL8*, but allopurinol seldom induces this pattern of CADR, whereas aminopenicillins are frequently involved in AGEP (Speeckaert et al. 2010). Another group, while studying

hepatotoxic drugs in THP-1 cells, observed an increase in mRNA expression and secretion of IL-8/CXCL8 after stimulation with terbinafine (Mizuno et al. 2010), another drug that frequently causes AGEP (Sidoroff 2012).

Nevertheless, as allopurinol, that most intensely upregulates this chemokine, seldom induces AGEP, we may suggest that the secretion of IL-8/CXCL8 by DC, keratinocytes or other tissue cells is not sufficient to influence the final pattern of the CADR, although it may significantly contribute to initiate the immune mediated adverse reaction.

G.5.5. Th1/Th2 polarization modulated by chemokines/cytokines induced by drugs

The pro-inflammatory cytokine IL-12, produced after DC exposure to contact sensitizers (Antonios et al. 2010), is important to drive the response into the predominant type 1 profile, involved in ACD and many delayed CADR (Martin et al. 2010). In our study, drugs differently modulated the expression of IL12B and CXCL10 genes, both related to a Th1 profile of immune response.

A significant stimulating effect was observed only for oxypurinol and sodium valproate, respectively on *CXCL10* and *IL12B*.

The expression of CXCL10 gene was downregulated by amoxicillin. This could be in agreement with the high frequency of betalactam-induced immediate reactions, which are promoted in an ambience rich in Th2 cytokines. However, we observed no upregulation of the Th2-promoting cytokine genes (IL-4 and IL-13), but this can also be due to the very low expression of these genes on THP-1, therefore allowing no definite conclusion.

Nevertheless, we may assume that, even though drugs have a maturational effect on dendritic-like cells, at this level, they do not seem to significantly influence T cell polarization.

G.5.6. I*n vitro* tests with drugs to predict their capacity to induce cutaneous adverse drug reactions

Like for contact sensitizers, the potency of drug-induced DC stimulation seems to correlate with their capacity to sensitize or induce severe CADR (Takenouchi et al. 2013).

In agreement with the clinical setting where allopurinol is responsible for the more severe CADR, allopurinol and oxypurinol that were the most potent chemicals in many of the end-points studied (p38 MAPKinase, *HMOX-1*, *IL-8/CXCL8*, *CXCL10* and cytotoxicity).

Aminopenicillins, that induced frequent but less severe CADR, also had a relevant effect, although less intense, on many of the activation markers (*HMOX-1, IL-8/CXCL8* and *CD40*).

On the other hand, the anticonvulsants carbamazepine and sodium valproate have a divergent behaviour in the clinical setting and in our THP-1 model. Carbamazepine, frequently responsible for CADR, was highly cytotoxic but only upregulated *HMOX-1* gene expression, whereas sodium valproate, seldom inducing CADR (Wang et al. 2012), had a more significant effect on *HMOX-1* and, also, on *CD40* and *IL12B*.

These results seem to contradict the low capacity of sodium valproate to induce CADR, but this drug has also shown somehow divergent results in another *in vitro* model to study the relation between DRESS and herpes virus reactivation. Sodium valproate was one of the more potent drugs to induce the in vitro activation of HHV-6, and other herpes virus, in T cells (Mardivirin et al. 2009),(Picard et al. 2010), whereas we have shown that all our patients with DRESS induced by carbamazepine tolerate this drug (Santiago et al. 2010).

The low effect of carbamazepine in THP-1 cells could be explained by the low metabolic capacity of these cells, by analogy with the similar *in vitro* results obtained with sulfamethoxazole that needs previous metabolism (Sanderson et al. 2007),(Elsheikh et al. 2010). Therefore, if carbamazepine behaves as a prohapten that needs bioactivation, carbamazepine itself may not be able to

activate THP-1 cells. We tried to test also carbamazepine exposide, one of the possible reactive intermediate metabolites, but results were also very poor. There are, nevertheless, arguments suggesting that carbamazepine needs no previous bioactivation. In a model using already sensitized T cells from patients, Chessman *et al.* showed that carbamazepine binds HLA molecules and directly activates drug-specific T cell clones (Chessman et al. 2008). Moreover, patch tests with carbamazepine are usually positive (Gonçalo and Bruynzeel 2011), suggesting carbamazepine is the active hapten or, eventually, that it is easily metabolized and processed by skin cells.

Otherwise, THP-1 cells may lack a receptor or a pathway important for the stimulation induced by carbamazepine. As shown in THP-1 cells, even strong or extreme sensitizers, like p-phenylenediamine and diphenylcyclopropenone, only activate one of the DC maturation markers under evaluation in the h-CLAT, and known sensitizers like isoeugenol, don't activate either CD54 or CD86 (Nukada et al. 2011). Several DC populations and several end-points in DC activation, associated with other in vitro assays, are necessary to evaluate the sensitizing capacity of chemicals (Kimber et al. 2011). Therefore, with a single DC line and a few end-points evaluated, we cannot take significant conclusions on the effect of carbamazepine on dendritic-like cells and its capacity to induce an innate response.

G.6. Conclusions

Our results show that drugs causing delayed CADR and contact sensitizers activate THP-1 cells in similar ways. Therefore, by analogy with ACD, drug induced xenoinflammation may act as a costimulatory second signal or as a co-adjuvant effect that favours HLA-drug-TCR recognition, enhances drug presentation and favours T cell sensitization.

Although, in CADR, an underlying inflammatory disease is considered to

deliver most of the necessary costimulatory signals (Hausmann et al. 2012), the recognition that the drug, by itself, also enhances an innate inflammatory reaction, may justify the occurrence of immune mediated CADR in the absence of other concomitant factors.

As these drugs create stress in dendritic-like cells, activate their detoxifying enzymes and induce the production of pro-inflammatory chemokines and cytokines, these "dangers signals" may "awaken" the immune system and promote an adaptive immune reaction against the drug or, eventually, also against other concomitant aggressors. In DRESS, induced for instance by allopurinol, reactivated virus or other drugs may also be the target of the adaptive immune response (Picard et al. 2010). Therefore, the pro-inflammatory effect induced by allopurinol or amoxicillin might also enhance the concomitant anti-viral T cell reaction.

Similarly to contact sensitizers, the intensity of the auto-adjuvant effect induced directly by drugs on THP-1 cells, with the drug promoting DC activation and, presumably, enhancing antigen presentation *in vivo*, may be somehow related to the frequency and severity of the CADR they cause. Allopurinol, that in clinical practice is one of the drugs most frequently involved in severe CADR (Halevy et al. 2008),(Mockenhaupt 2012), is also the most active drug inducing THP-1 cell activation. The effect can be compared to the very strong contact sensitizer, DNFB, in which concerns the pattern and intensity of gene expression modulation, particularly *HMOX-1* and *IL-8/CXCL8*.

Though somehow divergent, our results, combining several aspects of THP-1 activation, are promising concerning the identification of reliable parameters that might detect sensitizing properties of systemic drugs. Currently, to evaluate the sensitizing potential of chemicals to be used in contact with the skin, pre-marketing *in vitro* tests are proposed (Aeby et al. 2010),(Maxwell et al. 2011). Likewise, as a complementary safety measure, small chemicals

intended for use as systemic drugs could be tested in these models.

Both IL-8/CXCL8 and HMOX-1 are a sensitive and specific end-points in discriminating contact sensitizers from non-sensitizers. They are, at present, strong candidates to be included in *in vitro* assays for evaluating the contact sensitizing potential of chemicals and, eventually, also their potency as contact sensitizers (Ade et al. 2009),(Ainscough et al. 2013),(Mitjans et al. 2010), (Natsch 2010),(Neves et al. 2013). According to our results, IL-8/CXCL8 and HMOX-1 could also be recommended for further investigation in order to evaluate the sensitizing potential of systemic drugs, before their release into the market.

H.
FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

H. FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

H.1. Pathophysiologic mechanisms involved in cutaneous adverse drug reactions: relevance for clinical practice

The investigation of pathophysiologic mechanisms in CADR has been the object of many and remarkable studies in the last two or three decades and has brought many new insights into the knowledge of this very frequent but complex group of dermatological diseases. Moreover, as some CADR represent an immune reaction to a chemical that the organisms considers an aggressor, some aspects of the pathophysiology of the CADR can be translated to the understanding of how our skin responds to many other hazardous chemicals and living threats (virus, bacteria, fungi).

Also, as some CADR have a clinical expression that may resemble other skin diseases, the investigation of the CADR may also bring light into the pathophysiologic mechanisms of these related "idiopathic" skin diseases. As an example, the similarity between AGEP and pustular psoriasis, both at the clinical and histologic levels, has driven the investigation to study further the contribution of Th17 cells as well as the IL-36 pathway in both diseases, and also, in other cutaneous neutrophilic diseases (Tortola et al. 2012),(Lowes et al. 2013),(Navarini et al. 2013).

For the clinician, particularly for the Dermatologist, the knowledge of pathophysiologic mechanisms underlying each pattern of CADR is crucial for recognizing and understanding the very wide spectrum in their clinical expression, for performing an adequate search for the culprit(s) drug(s), for taking the relevant decisions during the acute management of the CADR, for the correct choice of complementary diagnostic tests to confirm the aetiology

of the CADR and, consequently, allowing a more correct patient advise concerning safer treatments in the future.

All these challenging aspects in CADR motivated our studies and encouraged us to share our enthusiasm with those working with us, in order to further improve our knowledge in this complex group of Dermatological diseases. We proposed to study only immune mediated delayed CADR, as they are the ones we observe most frequently during our practice as Dermatologists.

With histopathology we could understand similarities in the expression of effector mechanisms, particularly among DRESS and maculopapular exanthema and, also, in TEN, that can be similarly expressed in positive patch tests. *In vitro* studies unveiled a new aspect of the chemical reactivity of drugs that may be important, both during sensitization and elicitation of the CADR. Finding a significant risk factor for a severe CADR from allopurinol in our population, may create a roadmap for investing more in prevention than in treatment.

H.2. Histopathology in the diagnosis, prognosis or pathophysiology of DRESS

In delayed CADR, the maculopapular exanthema is the common initial presentation of many drug eruptions. Evolution from a less severe maculopapular exanthema to another exanthematous CADR with a poor prognosis, namely DRESS and SJS/TEN, may be predicted on clinical signs and laboratory data.

Nevertheless, clinical alarming muco-cutaneous and systemic signs and laboratorial changes are not specific, some occur relatively late and, consequently, escape the ideal timing to stop the culprit drug and initiate a more aggressive therapy. Therefore, there is an absolute need to find early markers of the progression of a maculopapular exanthema into a more severe form of CADR and establish early prognostic markers in these more severe

reactions. We intended to evaluate the contribution of the histopathology in the early diagnosis or, eventually, for establishing a definite diagnosis of DRESS.

H.2.1. The diagnosis of DRESS: histopathology and future diagnostic perspectives

Some delayed CADR may have very typical histopathology features when at their peak, namely AGEP, fixed drug eruption and SJS/TEN. Histopathology is much less typical at their beginning and there are no definitive specific histopathology aspects in exanthematous reactions that can confirm the clinical diagnosis of a CADR (Hari et al. 1999),(Bellini et al. 2013),(Seitz et al. 2013).

In which concerns the value of histopathology for the diagnosis of DRESS, and in agreement with the few other detailed histopathology studies in this CADR (Chiou et al. 2008),(Chen et al. 2010),(Natkunarajah et al. 2011),(Walsh et al. 2013), in our study of 15 patients, with skin biopsies collected at different time points in evolution, we found no specific histologic hallmark in the exanthema of DRESS that can definitively reinforce a clinical diagnosis.

We confirmed that histopathology in DRESS is highly variable, presenting as an intense pseudolymphomatous dermal infiltrate, a slight dermal and epidermal inflammatory infiltrate with lymphocyte or neutrophil exocytosis, a lichenoid infiltrate with vacuolization of the basal cells or with a significant degree of keratinocyte necrosis, almost reminding of TEN.

The more specific pattern is probably the pseudolymphomatous variant, that is not usually reported in other CADR, but which, in our experience, may occur only in long-standing DRESS. We observed a tendency to the increase of the density of the lymphocyte and eosinophil infiltrate in later exanthema. Therefore, this pseudolymphomatous pattern might occur too late and, consequently, with little value for an early diagnosis.

Although apparently more intense, many of the histopathology findings in DRESS are common to the those described in maculopapular exanthema in other studies (Naim et al. 2011), and none has been shown to be helpful to definitively distinguish DRESS from a maculopapular exanthema with no systemic involvement.

Interestingly, in the histology of positive patch tests, the dermal inflammatory infiltrate and the epidermal changes were more intense in DRESS than in the maculopapular exanthema, although with no definitive cut-off that allowed a perfect distinction between the two patterns of CADR.

We did not perform additional immunohistochemistry techniques but, by analogy with SJS/TEN, antibodies directed to targets related to the pathophysiology of DRESS, namely to granulysin and HMGB-1, might help in the early diagnosis and distinction between DRESS and maculopapular exanthema. Recently, Hashizume has found a very particular expression of HMGB-1 protein in the epidermis and dermis, along with the dermal accumulation of monomyeloid precursors, in 3 early skin biopsies of DRESS. Actually, in the epidermis of DRESS, HMGB-1 protein was not staining the nucleus of the keratinocyte, as in normal skin, but the cytoplasm, suggesting it might be preparing for secretion. Moreover, there was evidence of HMGB-1 expression at the whole epidermal thickness, a presentation that is very different from normal skin and from SJS (Hashizume et al. 2013). Further studies are needed to validate the sensitivity and specificity of this very particular aspect on the immunohistochemistry of DRESS, and its possible use as an early marker of the association of an exanthema with DRESS.

In the near future, to make a more clear distinction between a maculopapular exanthema, with no systemic involvement, and severe CADR, including DRESS, apart from histopathology, we will very probably have to rely on relatively refined laboratory tests that measure circulating levels of biomarkers that correlate with the activation of effector cells. On relatively recent research

studies, elevated serum concentrations of granulysin, soluble FasL, HMGB-1, as well as other alarmins, have been identified as possible good biomarkers to predict the evolution of a maculopapular exanthema to SJS/TEN or to DRESS (Nakajima et al. 2011),(Tohyama and Hashimoto 2012),(Schwartz et al. 2013b),(Hashizume et al. 2013). In SJS/TEN, serum granulysin is elevated during the initial phase of the exanthema, even before the mucosal erosions are present, and a cut-off of 10 ng/mL seems appropriate to distinguish between maculopapular exanthema and the more severe CADR (Abe et al. 2009),(Fujita et al. 2011). A rapid immunochromatographic test has been developed and, in 15 minutes, detects granulysin serum levels above 10 ng/mL. Although not yet fully tested, preliminary data has shown a good correlation with the ELISA techniques usually used, in research, to quantify granulysin (Fujita et al. 2011).

Therefore, in the future, the determinations of these serum biomarkers may be a very useful complementary test to perform in an initial phase of a drug-induced exanthema, particularly when it is associated with drugs often involved in severe CADR (allopurinol, anticonvulsants, cotrimoxazole or oxicams) or when the exanthema is associated with other symptoms (fever, malaise, oral or ocular discomfort).

Although it is very important to distinguish a maculopapular exanthema from DRESS, due to different therapeutic and prognostic aspects, this may not always be possible. Apart from the common histologic aspects observed in this study and in positive patch tests, somehow reinforced by similar clinical features previously referred, overlapping patterns between maculopapular exanthema with no systemic involvement and DRESS seem to exist (Gouveia et al. 2013), as suggested, also, by other authors (Bouvresse et al. 2012).

In an on-going study in our department, we are characterizing patients with these overlapping features, namely in which concerns clinical and histology parameters of the exanthema, presence and intensity of associated systemic manifestations and we intend to evaluate if these overlapping cases are related

with any specific drug, concomitant factor (viral infection) or HLA predisposing factors.

H.2.2. DRESS prognosis: histopathology and future prognostic markers

Histopathology has been used as an attempt to establish prognostic factors in CADR, mainly in SJS/TEN, but correlation between the intensity of the dermal infiltrate and the prognosis was not always evident in this CADR (Quinn et al. 2005),(Valeyrie-Allanore et al. 2013).

In the present work, some correlation could be detected between histopathology and severity of DRESS. There was a positive correlation between the intensity of the dermal-epidermal lymphocyte infiltrate and the severity of hepatic cytolysis and eosinophilia, and a weaker positive correlation between keratinocyte necrosis and hepatic cytolysis, the latter in agreement with the study by Walsh *et al.* (Walsh et al. 2013).

If the data of our study, only with 15 patients, is reproduced in larger studies, the histopathology of DRESS may be further used as a good prognostic marker. Nevertheless, we do not expect it will ever be a very strong prognostic marker. It will, certainly, be difficult to quantify the histopathologic changes and establish a cut-off to define the probability of a severe case. Moreover, histopathology changes seem to depend on the day of evolution of the DRESS, making this task even more difficult. Many more skin biopsies would be needed, performed in many patients, and at different days of evolution in the same patient, which is not feasible in a small centre like ours and might raise ethical issues.

To eventually reinforce the value of histopathology it might be interesting to correlate histopathologic aspects of DRESS and, also SJS/TEN, which might

have some prognostic relevance, with the determination of the serum concentrations of effector and skin damaging molecules, namely FasL, granulysin and HMGB-1. Actually, circulating levels of granulysin correlated nicely with disease severity, both in DRESS and SJS/TEN (Saito et al. 2012),(Abe 2008),(Schwartz et al. 2013b)(Hashizume et al. 2013).

Therefore, in a near future, and when and where these techniques are available, we may expect that circulating levels of granulysin or other effector molecules may be used more efficiently in severe CADR, both as early diagnostic, as well as prognostic markers.

H.2.3. Drug-induced or viral-related skin aggression in DRESS. Future diagnostic tests

We observed no specific aspect on the histopathology of DRESS that might be correlated with a specific culprit drug, namely when considering the main drugs causing DRESS in our study, allopurinol and carbamazepine. Differences might be expected, as these two chemicals differently activate relevant genes and signalling pathways in dendritic-like cells, as we observed in THP-1 cells. As these differences probably occur only at the initial innate immune reaction and this represents only the "awakening" of the immune system, we may understand that the final effector phase evaluated at histopathology, shares aggressive pathways that do not significantly depend on the initial innate reaction.

In the six patients within the study in whom we looked for the presence of circulating DNA for HHV-6, we found no significant histopathology difference between positive and negative cases. Nevertheless, we noticed that 2 out of the 3 cases with confirmed HHV-6 reactivation were the only cases, among the 15 studied, who had a particular concentration of lymphocytes of the

inflammatory infiltrate around the adnexa, particularly around the follicles. We have no explanation for this distribution of the infiltrate, as there is no report of the viral localization to the follicles.

A future complementation of the classical histology with in situ hybridization, to localize the viral genome, and/or immunohistochemistry, to co-localize the viral antigen and infected T cells, might contribute to understand better the real localization of the virus in the skin and, hence, explain if the this histopathology pattern with perifollicular distribution, for which we found no previous detailed description, has any plausible explanation.

Moreover, the detection of the viral genome in skin cells and their correlation with histopathology changes might contribute to understand the importance of the immune anti-viral response in skin lesions of DRESS. Other studies have already shown the presence of the antigens or the genome of HHV-6 and other herpes virus in affected organs, including the skin (Hashizume et al. 2013). Moreover, T cells that infiltrate the lesions carry a TCR that recognizes mostly herpes virus (Picard et al. 2010),(Ozcan et al. 2010), also reinforcing an important place of the anti-viral response in the pathophysiology of DRESS.

In analogy to DRESS, a very similar syndrome responding to antiviral therapy has been described in HIV-1 infected patients with low $CD4^+$ counts (Sbidian et al. 2010). This rare syndrome presents as a severe chronic erythroderma resembling mycosis fungoides, lymphadenopathy and severe eosinophilia, as in DRESS, and, on histology a pseudolymphomatous T cell infiltration, interface dermatitis or epidermal lymphocyte exocytosis, mostly with polyclonal or oligoclonal $CD8^+$ T cells that recognized HIV-1. This long-lasting erythroderma showed a complete or almost complete response to HAART (highly active anti-retroviral therapy) in a very significant number of cases (Sbidian et al. 2010). By analogy with the erythroderma in AIDS that responded to HAART, a documented viral reactivation in DRESS may support the use of specific anti-viral therapies, namely ganciclovir and valganciclovir which were also shown

to ameliorate DRESS in a reduced number of cases (Criado et al. 2012),(Moling et al. 2012).

Viral reactivation in DRESS seems unquestionable, although different virus may be involved and at different time points. Confirmation of viral reactivation, particularly of HHV-6, is one of the Japanese criteria for the definitive diagnosis of DRESS (Shiohara et al. 2007). Serology with an increase in IgM antibody titters can be helpful, but detection of viral copies in the blood is the ideal test to confirm viral reactivation, although this seems to occur particularly at the 2nd or 3rd week of evolution. Recently, based on the fact that most herpes virus involved in DRESS are localized in the oropharynx in their latent status, a quantitative PCR analysis of saliva was compared to the blood PCR. This test was shown to be helpful to document viral infection and viral shedding, with a good correlation with circulating viral loads, but only for HHV-6 and CMV. Viral shedding for HHV-7 and EBV in the saliva was rather unspecific, with many positive reactions in controls (Descamps et al. 2013)

As it has been shown that some drugs have the capacity to promote reactivation and proliferation of latent herpes virus within lymphomononuclear cells, at least *in vitro*, it would be mandatory to identify all drugs that, like amoxicillin, promote such viral reactivation and have been reported to aggravate DRESS (Mardivirin et al. 2010), as also occurred in three of our patients. Nevertheless, in vitro results have to be correlated with clinical data. Sodium valproate, one of the most active drugs in promoting viral replication *in vitro* (Mardivirin et al. 2010), is regularly used by us and other groups as the alternative safe drug in DRESS induced by aromatic anticonvulsants (Santiago et al. 2010).

Therefore, when the drugs that promote viral replication are correctly identified *in vitro* and correlated with a relevant *in vivo* effect in DRESS, these drugs should be completely avoided until full recovery of the syndrome. Promoting viral proliferation, particularly during severe immunosuppression,

will allow infection of resident skin cells or skin infiltrating cells, which will be the target for an active anti-viral immune response, particularly during immunoreconstitution. This might explain, for instance, an increased occurrence of herpes zoster after DRESS recovery (Kano et al. 2012), as observed in one of the patients of this study.

At present, it is difficult to know the relative participation of the anti-viral and anti-drug immune reaction. A specific anti-drug response is documented in lymphocyte stimulation tests (LST) and skin tests with drugs, particularly in DRESS induced by carbamazepine (Santiago et al. 2010). Nevertheless, variability in immunocompetence during the course of DRESS, may be responsible for a negative LST in the initial acute immunosuppressive phase and the test will become positive only after recovery (Kano et al. 2007).

We have no experience with LST variability in DRESS. By analogy, we tried to perform patch testing in skin areas free from the exanthema, during the acute phase of DRESS to compare with the results after resolution of the CADR, which we know are positive in many cases (Santiago et al. 2010). But it is not easy to interpret the patch test results during the acute state, mainly due to the on-going exanthema and the involvement of tested areas. Therefore, with patch testing, we could not document this state of "non-response" during the acute phase of DRESS.

Opposing the immunosuppressive phase, immunorecovery after resolution of DRESS may favour the development of autoimmunity against targets outside the skin. It is therefore mandatory to search for autoantibodies during and after DRESS recovery (Descamps 2013),(Cookson et al. 2013). Anti-thyroid antibodies are found more frequently, often with thyroid disease (Cookson et al. 2013), but we did not find them in our patients, until now (data not shown). Probably, the regular use of corticosteroids in moderate to high doses during the acute phase, in our patients, may have hindered the development of autoimmune phenomena, as recently suggested.

H.3. Patch testing to confirm aetiology of the adverse reaction and study pathophysiologic mechanisms

There is no unequivocal test to confirm the diagnosis of the culprit drug in delayed CADR. Drug rechallenge, usually considered the gold standard test, has important limitations, particularly in severe CADR, and is not standardized (Schnyder and Pichler 2012). *In vitro* techniques (LST or the ELISpot) are not widely available and their sensitivity and specificity are still far from desirable. Therefore, *in vivo* skin tests, adapted for delayed CADR, like the patch test, can be safely used after resolution of the reaction. When negative, intracutaneous tests with late readings can be performed, even though with restrictions in severe CADR. Moreover, they do not very significantly improve the sensitivity of the patch test, namely in the case of penicillins (Romano et al. 2013).

Although difficult to ascertain definitively, due to the absence of a positive control or a gold standard diagnostic test, sensitivity of patch tests is rather low and depends much on the drug and on the pattern of CADR (Barbaud 2009),(Gonçalo and Bruynzeel 2011),(Schnyder and Pichler 2012). No significant improvements in skin testing efficiency are expected in the near future, except for the possible use of new vehicles that improve drug penetration and an increase in commercially available preparations to perform patch tests or standardized, sterile and long lasting solutions that might be made available for intracutaneous tests, in a more generalized practice.

Patch test specificity is questioned mainly when, in the absence of pure chemicals in standardized vehicle and concentration, patch tests are performed with the commercial drugs. Unhappily, this has to be done too often, as there are very few commercialized compounds for patch testing, compared to the number of drugs frequently inducing delayed CADR.

Therefore, skin tests have to be used, just as one of the possible useful diagnostic tools, probably the first to be used, due its safety. Moreover, it can have some additional advantages.

As shown in our study, patch testing can also be of value in studying cross-reactions. When positive, cross-reactive patch tests may be important for patient advise, concerning extended drug avoidance. On the other hand, negative tests to analogous substances may help orient drug provocation tests, using drugs that caused no reaction on the patch test, in order to confirm their safety.

Apart from diagnostic purposes, we showed that patch testing can give an important contribution to study pathophysiologic mechanisms involved in CADR.

H.3.1. Histopathology of the patch tests compared with the histopathology of the acute cutaneous adverse drug reaction

Histopathology of positive patch tests showed an inflammatory lymphoid dermal-epidermal infiltration with epidermal aggression, occasionally with vesicles as in ACD, pustules as in AGEP or full-blown epidermal necrosis as in TEN. This type of reaction, simulating delayed hypersensitivity reactions, is a strong argument to reinforce the specificity of the patch tests as an aetiology diagnostic tool. This was also evident even when patch testing was performed using a fresh preparation with the powder of patients' pills, namely in the case of DRESS from abacavir.

The epicutaneous application of the chemical in the patch test reproduced very clearly the skin reaction pattern of CADR, often both at the macroscopic and microscopic level. This was particularly evident in AGEP. As shown previously, macroscopic pustules were observed, particularly after the patch test reading at day 3 and, in histopathology, neutrophil exocytosis and intraepidermal or subcorneal pustules could be clearly identified (Britschgi et al. 2001),(Britschgi and Pichler 2002),(Barbaud 2009),(Serra et al. 2011),(Serra et al. 2012)..

Moreover, we could show, in a positive patch test from carbamazepine in TEN, a positive Nikolsky's sign and a full thickness epidermal necrosis, the histologic hallmark of TEN. We found no such macroscopic or histopathology description on a positive patch test from TEN, eventually also because patch testing is not so frequently positive in this CADR (Wolkenstein et al. 1996).

In positive patch tests in DRESS we observed, in one case, a pseudolymphomatous reaction, as was usually described in the acute exanthema in the initial publications of DRESS (Husain et al. 2013). This case showed a dense lymphoid inflammatory infiltrate, forming lymphocyte microabscesses in the subcorneal layer, as in mycosis fungoides, which we could not observe in the biopsies from the exanthema in other DRESS cases, even in the patient with the pseudolymphomatous pattern in histopathology.

In patch tests from the exanthematous reactions, there was a relative absence of eosinophils, cells that are considered a marker for drug hypersensitivity, although, very probably, erroneously (Seitz et al. 2013). Eosinophils were, nevertheless, present in 80% of the exanthema of our DRESS patients, although in low amounts, and in 60% of patients with maculopapular exanthema, in the study of Naim *et al.* (2011).

In general, and as might be expected by analogy with the acute reaction, the intensity of the inflammatory infiltrate and epidermal aggression, with vesicle formation, increased progressively from the positive tests in maculopapular exanthema, to the positive patch tests in DRESS and to SJS/TEN, but with no clear cut difference between the different CADR.

The continuum in the intensity and morphology of changes observed within patch test biopsies from several exanthematous reactions, in association with similarities in their respective clinical setting, may reinforce the previously discussed notion that there is some overlap between CADR patterns, particularly between the maculopapular exanthema, DRESS and SJS/TEN, although these are mostly very well defined phenotypes of delayed CADR (Pirmohamed et al. 2011),(Bouvresse et al. 2012).

H.3.2. Patch test histopathology to study effector pathomechanisms

The analogy of the histopathology between the patch test and the skin during the acute eruption in CADR can support the use of patch testing as a good tool for studying pathomechanisms involved in the effector phase of the CADR. Having a similar aggressive machinery, T cells recruited and induced to proliferate specifically at the patch test site by the presence of the drug will, very probably, induce an effector response that shares many aspects of the acute reaction.

Skin testing, performed both in patients and experimental animals, has been important to characterize the importance of $CD8^+$ effector T cells involved in the effector phase of nonimmediate CADR (Rozières et al. 2009b),(Rozières et al. 2010). In two cases, by immunohistochemistry, we also confirmed the presence of T cells, mainly $CD8^+$, in the dermis and epidermis of patch tests, but we did not characterize the phenotype of infiltratory cells in most of the patch tests.

We have not studied antigen specificity of the T cells infiltrating patch tests, but, in other studies, drug specific T cells were isolated from positive patch test reactions and showed the same phenotype as T cells isolated from the skin or the circulation during the acute eruption (Yawalkar et al. 2000). Recently, in a positive patch test from tetrazepam in a case of AGEP, high levels of mRNA for IL-8/CXCL8 were observed (Thomas et al. 2008), confirming similar effector pathways in the acute eruption and the patch test and, consequently, reinforcing the similarity in clinical and histopathology changes, in the two settings.

By analogy with ACD, further studies performed in the different stages of evolution of the patch tests, including the evaluation of the phenotype and transcription factors of infiltrating cells, as well as their TCR repertoire, and

the characterization of the cytokines and chemokines present in the patch tested skin, may certainly bring more knowledge, both on the initial steps and, later, on the effector mechanisms involved in delayed CADR.

H.4. Genetic risk factors for severe cutaneous adverse drug reactions

In pharmacogenomics, the association of a genetic marker with an increased risk for CADR is an active area of research (Wei et al. 2012c),(Hershfield et al. 2013). It has already resulted in very relevant data that allowed the prevention of many severe and life-threatening CADR, but also contributed to understand their pathophysiology.

At present, the association of these CADR mostly with HLA-class I molecules, reinforces the importance of the participation of drug specific $CD8^+$ T cells (Rozières et al. 2010). These T cells recognize the drug bound to HLA-class I molecules, both during antigen presentation and during the destruction of cellular targets that carry the drug (Chessman et al. 2008),(Bharadway et al. 2012).

The identification of HLA molecules as risk factors for specific drugs, has also encouraged other studies to understand how drugs, or their metabolites, bind aminoacids in the HLA antigenic cleft and are recognized by T cells (Bharadway et al. 2012),(Bell et al. 2013).

H.4.1. HLA-B*58:01 as a risk factor for DRESS in a European population

In the present work, the association between HLA-B*58:01 and allopurinol-induced DRESS was reported for the first time in European patients, although

the association with SJS/TEN was already known in Europeans (Lonjou et al. 2006),(Lonjou et al. 2008).

The association between HLA-B*58:01 and severe CADR induced by allopurinol, known since 2004 (Chung et al. 2004), has a very peculiar variability in which considers ethnicity. The risk, both for DRESS and SJS/TEN, is very high in Han Chinese (Somkrua et al. 2011),(Jung et al. 2011),(Cao et al. 2012), but much lower for Japanese, Australian and European patients with SJS/TEN (Lonjou et al. 2008),(Kaniwa et al. 2008),(Lee et al. 2012b). The risk for severe CADR, both DRESS and SJS/TEN, determined in our patients (OR = 88.5 with a 95% CI: 37.60-207.92) is within the values reported for SJS/TEN in other European populations (Lonjou et al. 2006),(Lonjou et al. 2008).

The prevalence of HLA-B*58:01 in the normal population is much higher in Han Chinese (± 15%) than in Europeans (<2%) or Japanese. But, the different HLA prevalence is certainly not the answer for such a difference in risk, that reaches an OR = 348.88 in Han Chinese. Probably, as shown for carbamazepine, specific TCR sequences are also needed for the recognition of the drug combined with HLA. Also, eventually, along the centuries and determined by different exposure to pathogens in Han Chinese, there was some beneficial positive selection of T cell clones carrying TCR that recognize relevant pathogens and, unexpectedly, also allopurinol or its metabolites. Other possible explanations for different strength of the risk association in the distinct populations, include epigenetic modifications induced by environmental chemicals, different metabolic machinery or particular SNPs in linkage disequilibrium with HLA-B*58:01, and that are present only in one of the populations, and which may hinder, or favour, the risk for severe CADR from allopurinol.

H.4.2. HLA-B*58:01 genotyping in the prevention of severe allopurinol-induced CADR

Other stronger HLA associated risk factors for severe CADR were defined, especially for carbamazepine-induced SJS/TEN in Han Chinese (HLA-B*15:02) and abacavir-induced hypersensitivity syndrome worldwide (HLA-B*57:01). The associated risk and the negative and positive predictive factors were much stronger for these drugs and cost-effectiveness of HLA pre-testing before initiating therapy was easily established.

HLA-B*57:01 has a positive predictive value around 55% and a negative predictive value near 100% for abacavir hypersensitivity. Pre-testing, accepted worldwide, almost completely abolished this CADR (Phillips et al. 2005),(Hughes et al. 2008),(Phillips et al. 2011),(Hashizume 2012).

Also, there is a 2500-fold increased risk of carbamazepine-induced SJS/TEN in Han Chinese carrying HLA-B*15:02, with a 92% sensitivity and 96% specificity (Yang et al. 2007),(Hashizume 2012). Therefore, pre-evaluation for HLA-B*15:02 in now mandatory and financed by the National Health Insurance in Taiwan and it has abolished carbamazepine-induced SJS/TEN in this country (Chen et al. 2011),(Wei et al. 2012b),(Maekawa et al. 2012). Such a strong risk factor associated with HLA-B*15:02 is not observed for DRESS, neither for severe CADR in Europeans nor in Japanese patients. In these populations, HLA-A*31:01 represents a relative risk for carbamazepine-induced DRESS and SJS/TEN (McCormack et al. 2011),(Lichtenfels et al. 2014), as observed in some of the patients included in this study. Nevertheless, as the risk is not as high, regular genotyping before therapy is not so strongly recommended, although recently suggested (Amstutz et al. 2014a),(Amstutz et al. 2014b), particularly with new rapid techniques available to detect this allele (Uchiyama et al. 2013).

For allopurinol, although rapid and cheaper techniques are also being developed to detect HLA-B*58:01 (Kostenko et al. 2011),(Maekawa et al. 2012), the lower risk factor assessed and the low prevalence of this HLA in our

population (± 2%), question the cost-effectiveness of pre-genotyping for HLA-B*58:01 before allopurinol use.

It was our intention to perform an economic study to evaluate the cost of the medical intervention in the severe CADR from allopurinol, which combined with the indirect costs of the days out of work and the possible cost of the sequelae. This would allow us to calculate if pre-testing could be cost-effective for the whole population needing allopurinol, only for patients at higher risk or, if it is not cost-effective at all. Nevertheless, the suffering and mortality of these patients with severe CADR from allopurinol should not be decided only on a question of cost, and preventive measures are absolutely needed to reduce the number and severity of allopurinol-induced CADR.

H.4.3. Future perspectives in the evaluation of genetic risk factors

As previously referred, patch testing that significantly contributed to establish the strength of association between abacavir and HLA-B*57:01 (Phillips and Mallal 2009a), is usually negative with allopurinol (Vieira et al. 2004a),(Santiago et al. 2010). If this was not the case, patch testing could be extremely important to refine the association observed between HLA-B*58:01 and severe CADR from allopurinol. In our 25 patients, the imputability index was very high, as allopurinol was the only or one of the few drugs recently introduced. But, it would have been important to have a complementary test to confirm allopurinol as the culprit drug, when studying its association with genetic markers.

Moreover, it might be important to extend this relation to other patterns of CADR, apart from SJS/TEN and DRESS, and find other risk factor that might help us prevent this and other severe CADR induced by other drugs.

GWAS have been performed with a significant number of European patients with SJS/TEN, but the only significant association was in chromosome 6 and related to HLA, in a certain way, confirming previous studies of the association of HLA-B*58:01 and allopurinol (Génin et al. 2011) and of HLA-A*31:01 and carbamazepine (Lichtenfels et al. 2014). It will certainly be difficult to retrieve significant results, even with multicentre studies. CADR do not represent a single disease and, even for one single pattern of CADR, the risk factors are mostly drug dependent, which can probably justify the low yield of the European study conducted in SJS/TEN, induced by different drugs. Therefore, it may be extremely difficult to find, outside large multicentre studies, a sufficient number of cases with the same pattern of CADR and from the same drug, in order to have significance in GWAS. Nevertheless, with the present multicentre collaboration that has been implemented in Europe and Asia, namely with the extension of RegiSCAR, and also in America and Australia, it is possible that GWAS will, eventually, find other important and relevant genome associations.

In a very recent paper, still without using this technique, Barbaud *et al.* showed the association of DRESS, in the French population, with cytokine gene polymorphisms that may enhance the inflammatory cascade. The association of polymorphisms in IL-1β-511C and in the IL-1RA (receptor antagonist) act synergistically for a more intense pro-inflammatory effect, and IL-10 gene polymorphism is associated with a low production of this anti-inflammatory cytokine. These cytokine gene polymorphisms observed in DRESS, but not in patients with maculopapular exanthema, need to be validated in larger studies (Barbaud et al. 2013b).

H.5. I*n vitro* response of dendritic-like cells to systemic drugs: implications on pathophysiology of cutaneous adverse drug reactions

Dendritic cell activation, widely evaluated *in vitro* with contact sensitizers, and relevant during the sensitizing process in ACD, has seldom been systematically studied with drugs causing CADR, with the few exceptions of penicillins and sulfamethoxazole (Rodriguez-Pena et al. 2006),(Sanderson et al. 2007),(Sebastian et al. 2012).

Using THP-1 cells, frequently used for evaluating the sensitizing potential of contact sensitizers and also, tentatively, their potency as contact sensitizers (Ashikaga et al. 2006),(Aeby et al. 2010),(Nukada et al. 2011),(Neves et al. 2013), we observed that, like contact sensitizers, systemic drugs studied exert some cytotoxicity but, in conditions of high cell viability (>70%), they activate relevant genes and signalling pathways in these dendritic-like cells.

In this *in vitro* model, modification of gene expression coding for different markers of DC activation was dependent on the drug studied, in a similar way as it also varies with different contact sensitizers. Moreover, the intensity of the response for each gene also varied with the drug. It was interesting to recognize that allopurinol and its active metabolite, oxypurinol, were almost as effective as the very strong contact sensitizer, DNFB, in upregulating expression of some the genes studied, namely *HMOX-1* and *IL-8/CXCL8*.

The similarity of the *in vitro* response to systemic drugs that cause CADR and to topical chemicals that cause ACD, seem to confirm the existence of another common step in the pathophysiologic mechanisms in these two types of immune mediated delayed cutaneous reactions. This might be expected, as both ACD and delayed CADR are caused by small molecular weight chemicals that haptenize proteins and are recognized by specific T cells, which induce skin aggression to remove the “offending” allergen bound to skin cells (Martin et al.

2010),(Peiser et al. 2012). Moreover, often the same chemical can interchange in their exposure either as contact sensitizers or a systemic drug. Systemic drugs can behave as contact allergens when they are manipulated in an occupational setting (Vander Hulst et al. 2010),(Landeck et al. 2012),(Kerr et al. 2008) or just by "proxy" exposure (Baeck and Goossens 2009),(Teixeira et al. 2013), and contact allergens can arrive in the skin through systemic route and induce systemic contact dermatitis, with clinical expressions simulating CADR, as in SDRIFE (Bircher 2012). This occurs namely with ingested spices that share antigens with perfumes, with metals (nickel, gold) liberated from body implants that reach the skin after systemic distribution or with airborne allergens absorbed through the skin or the respiratory mucosae (Veien 2011).

H.5.1. Significant and relevant upregulation of genes related to oxidative stress (*HMOX-1*) and *IL-8/CXCL8*

One of the most intensely upregulated genes in this model was the *HMOX-1* gene that codes for a cellular detoxifying enzyme, dependent on the Keap-Nrf2-ARE pathway, that cells use in response to oxidative stress. Moreover, the gene for *IL-8/CXCL8*, a chemokine secreted in response to cellular stress and dependent on a similar pathway, was similarly upregulated by several drugs.

This suggests that systemic drugs cause stress in contact with living cells that, *in vivo*, may be relevant to initiate an innate inflammatory response by causing the production of "danger signals". These signals, also known as "alarmins", produced at the place where the drug localizes or is metabolized in our body, namely in the skin, may secondarily activate the adaptive immune response by potentiating the activity of antigen presenting cells or effector cells. Moreover, this innate response may, eventually, contribute to attracting inflammatory cells to the skin, or any other organ where the drug may co-localize, as in AGEP where IL-8/CXCL8 attracts neutrophils to the skin (Kabashima et al. 2011).

Cellular stress caused by drugs, *in vitro,* may also be relevant in other pathophysiologic aspects of CADR. It was shown, particularly with sulfamethoxazole and under stressful *in vitro* situations (increased temperature, presence of bacterial products, costimulatory chemicals or necrotic cells), that the culprit drug combines more actively with soluble or cellular proteins and forms significantly more adducts, the backbone for T cell recognition. This facilitates drug presentation by professional cells and also increases the number of available targets, during the effector phase (Lavergne et al. 2009),(Elsheikh et al. 2011). I*n vivo*, during infection or inflammatory conditions that increase body temperature, augment pro-inflammatory cytokines or cause cell death to liberate significant amounts of DAMP, the formation of more relevant drug/protein adducts may occur, as shown in cystic fibrosis patients exposed to penicillins. This may be one possible explanation, apart from the higher exposure, for the more frequent CADR from these antibiotics in patients with cystic fibrosis (Elsheikh et al. 2011),(El-Ghaiesh et al. 2012).

Moreover, the formation of drug adducts can also stimulate the innate immune system in other ways. Drug adducts with HLA class I molecules, may significantly modify these HLA molecules and prevent the usual function of inhibitory receptors in NK cells. Under these circumstances, cells with a drug-modified HLA can be potentially targeted and killed by the innate NK cells, which also secrete cytokines, like IFN-γ, that will further recruit T cells. The Spanish group studying drug eruptions and evaluating the innate response in CADR, has shown that amoxicillin spontaneously forms adducts with the heavy chain of HLA-I molecules and hinders the effect of inhibitory receptors in NK cells (Morel and Bellón 2007). Consequently*, in vivo*, NK can kill the target cell combined with the drug and secrete cytokines that initiate or potentiate the antigen presentation or effector function. In CADR, these NK cells have been observed in high numbers, particularly in areas with extensive keratinocyte necrosis (Bellón and Blanca 2011),(Schlapbach et al. 2011b). Although we did not stain for these NK cells of the innate immunity, we found similar aspects of epidermal damage in skin histology sections, both from the

exanthema in DRESS and from positive patch tests in the more severe drug reactions.

H.5.2. Upregulation of genes related to phenotypic markers of dendritic cell maturation

THP-1 cells exposed to some systemic drugs, namely ampicillin and sodium valproate, upregulated genes for DC maturation markers (*CD40* and *CD83*) although not so significantly as LPS. We presume this direct effect of the drugs on DC maturation also occurs *in vivo*, namely in skin DC or other circulating or tissue antigen presenting cells that come in contact with the culprit drug, and, therefore, it is expected that, by maturating and activating the relevant antigen presenting cells, drugs also directly potentiate their own presentation to the immune system, eventually overriding the absence of external costimulant factors, usually considered necessary in CADR.

Penicillins, sulfamethoxazole and clofibrate have also been shown to upregulate DC maturation markers, namely CD40, CD86 or CD83, both in THP-1 and other cells lines.

Interestingly, when using circulating monocyte-derived DC, upregulation of DC maturation markers on exposure to penicillins occurred only in cells from penicillin allergic patients (Rodriguez-Pena et al. 2006). Moreover, only the NK cells from amoxicillin allergic patients (not from controls) were able to increase their cytotoxic potential and the release of INF-γ and to activate the crosstalk with DC (Chaves et al. 2010). These cells, moDC and NK cells, which belong to the innate immune system, presumably without an individualized response and without memory, were supposed to respond in a similar way to the presence of the drug, independently of a previous sensitization. But, this may not be the case.

Therefore, still unexplored differences may exist between individuals at the level of the innate response, that may dictate those who develop, or not, DC or NK cell activation and, seemingly, the CADR. This deserves further study, as some of the most significant risk factors for initiating sensitization may reside within the initial innate response to the drug. Future studies may find in the innate response many strong determinants on which risk factors will rely, at least in the absence of costimulatory factors.

H.5.3. Intracellular signalling pathways involved in dendritic cell activation

Most drugs activated p38 MAPKinase, a signalling pathway usually involved in DC maturation and cytokine production (Arrighi et al. 2001),(Kagatani et al. 2010),(Neves et al. 2013). The drug that mostly activated p38 MAPK (oxypurinol) was not associated with an upregulation of the maturation markers *CD40* or *CD83*, but was the most potent on upregulating *IL-8/CXCL8*, also dependent on this signalling pathway (Kagatani et al. 2010),(Martin 2012).

Significant p38 MAPK was evident only for oxypurinol and not as intense as the strong sensitizer, DNFB. This signalling pathway, used for many contact sensitizers and considered an important step in initiating the innate immune response that favours sensitization, may not be the single intracellular pathway involved in DC activation by drugs.

As shown for chemicals identified as contact sensitizers, systemic drugs may activate DC through other potential pathways, namely direct activation by the chemical itself, by hyaluronic acid fragments or ROS and through TLR-4/MyD88, TLR-2/TLR-4/MyD88 or ATP receptors which, consequently, activate different signalling pathways and transcription factors (MAPK, NF-κB, Keap-1/Nrf2/ARE or the NLRP3/caspase-1 inflammasome) (Martin 2012). Use of different intracellular signalling pathways may justify different outcomes in

DC activation that, in the present study, we evaluated only at the gene level not at the expression of the correspondent protein.

Therefore, to understand the whole processes of DC activation and the more complete process of antigen presentation, it would be necessary to study all these and, eventually, other signalling pathways, other relevant genes and also effector proteins which were not included in our study. The addition of specific inhibitors of these pathways to the culture medium might also be important to understand the relevant pathways used by systemic drugs to activate DC. Moreover, some of these inhibitors might, eventually, have some clinical applicability.

H.5.4. Correlation between the intensity of the *in vitro* and *in vivo* effects of systemic drugs

In agreement with the frequency they induce severe CADR, allopurinol and carbamazepine showed the most cytotoxicity in THP-1 cells, but carbamazepine was relatively weak on the activation of these cells.

Allopurinol particularly induced *HMOX-1* and *IL-8/CXCL8* gene upregulation, with an intensity very similar to the strong sensitizer, DNFB. Although oxypurinol is considered the responsible metabolite, both chemicals exhibit a similar effect, suggesting that allopurinol may be easily transformed in THP-1 cells or it may also act as the active sensitizer (Yun et al. 2014).

The reduced stimulating effect of carbamazepine on THP-1 cells may be due to the very poor metabolizing machinery of these cells, the argument used to explain the absence of effect of sulfamethoxazole on a similar model, as sulfamethoxazole metabolites are considered responsible for the immune reaction (Elsheikh et al. 2011). Unexpectedly, sodium valproate, which seldom induces CADR, was rather intense in the upregulation of genes coding for DC maturation markers *CD40* and *CD83* and the Th1 driving cytokine, IL-12, namely the particle p40 shared by IL-12 and IL-23.

Like in similar DC models, which showed variability in the response to the different penicillin derivatives (Rodriguez-Pena et al. 2006),(Sebastian et al. 2012), the two aminopenicillins showed a modest response in THP-1 cells, generally a stronger response for ampicillin, that upregulated *CD40, HMOX-1* and *IL-8/CXCL-8*. In agreement, in the h-CLAT using also THP-1 cells, penicillin G activated CD54 but not CD86 and was, therefore, considered a weak sensitizer (Nukada et al. 2011). This may be in agreement with the relatively low number of delayed reactions from penicillins, particularly of severe CADR, if we consider their very wide use and the frequent incorrect attribution of delayed skin rashes to aminopenicillins, when their real cause is a viral infection (Schnyder and Pichler 2012),(Seitz et al. 2013).

We studied a very limited number of drugs, some of which may behave as prohaptens. Therefore, we cannot know how most systemic drugs that induce immune mediated delayed CADR behave in this *in vitro* model. We know even less about the drug-induced innate pro-inflammatory activity *in vivo,* which we could only infer, based on similarities with ACD, as we performed no animal or *in vivo* studies.

As a more proxy study, we could use skin explant models or co-cultures of DC and keratinocytes, as these latter cells, that represent the majority of epidermal cells, may certainly play an important role in the innate response and can, very probably, influence skin DC response, as shown in ACD (Pichowski et al. 2001),(Rustemeyer et al. 2011).

Although we studied a limited number of drugs and end-points in THP-1, activation of these cells that have characteristics of antigen-presenting cells can be translated, *in vivo*, into the enhancement of the DC activity and, therefore, favour drug sensitization in CADR.

H.5.5. I*n vitro* methods to predict the sensitizing potential of systemic drugs

At present, animal studies are not allowed to study the skin sensitizing potential of chemicals to be used further in cosmetics and several in vitro tests are used to study new chemicals, but there is no single *in vitro* test that is able to detect all sensitizers.

Like in our study with systemic drugs, where THP-1 cells showed almost no response to carbamazepine, when these cells are tested with contact sensitizers, namely in the h-CLAT, they do not show the same activating response to all chemicals. For instance, potent sensitizers like p-phenylenediamine and methylchloroisotiazolinone/methylisothiazolinone activate CD86 but not CD54, dyphenylcyclopropenone activates CD54 but not CD86 and, the known sensitizers, isoeugenol and hydroxypropylmethacrylate do not activate any of these markers in THP-1 cells (Nukada et al. 2011).

Therefore, to discriminate between sensitizers and non-sensitizers and, eventually, to quantify the potency of sensitizing chemicals, it is now strongly advised to use and combine the results of the different tests, using several end-points of DC activation and different DC-like cells lines in conjunction with other *in vitro* models that study skin bioavailability, hapten chemistry, peptide binding, skin metabolism and T cell activation (Aeby et al. 2010),(Kimber et al. 2011),(Maxwell et al. 2011),(Takenouchi et al. 2013),(Neves et al. 2013),(Hirota et al. 2013).

In which concerns safety of systemic drugs, animal and humans studies are still used, but our experiments with THP-1 cells can suggest to use this model, in conjunction with others, to test the sensitizing potential of systemic drugs and, therefore, evaluate their potential to induce CADR. This might, eventually, be used before animal testing and before the introduction of drugs into the market.

H.6. FINAL REMARKS

The studies presented in the previous 4 chapters of this thesis are in continuation of the work developed by our group, during the last 2 decades, on the understanding of the pathophysiologic mechanisms involved in delayed immune mediated CADR and, consequently, on the improvement of diagnostic and preventive measures. They have cleared a few aspects but have raised new questions that are already being studying and whose results will be presented in the near future.

Our modest contribution to the knowledge of pathophysiologic mechanisms in delayed CADR includes some aspects of basic research which confirm, for the first time for some systemic drugs, that they induce an innate immune reaction similar to contact sensitizers, expressed *in vitro* in THP-1 cells mainly by the upregulation of *HMOX-1, IL-8/CXCL8* and *CD40* gene expression and activation of p38 MAPK. Dendritic-like cell activation may be relevant in the process of antigen presentation and contribute to "wakening" the adaptive immune system to mount a specific anti-drug T cell reaction. It is our intention to extend this study to other drugs, to other genes and signalling pathways and to co-cultures with keratinocytes and T cells and, also, to introduce new conditions in skin cultures that reflect general inflammation (increased temperature, co-exposure to microbial products or stressful molecules) and evaluate how they may interfere with the process of activation of dendritic-like cells.

In severe CADR from allopurinol, we confirmed the risk of HLA-B*58:01 carriers in developing SJS/TEN, already reported in several population groups. Moreover, we showed that this HLA genotype also confers a significant risk for DRESS (OR=85.36) in Portuguese patients, which had not been previously shown in European populations. We could not, nevertheless, establish the cost-effectiveness of HLA typing before prescribing allopurinol.

Histopathology of DRESS during the acute reaction and histopathology of positive patch tests, although in a limited number of patients, helps to understand effector mechanisms in delayed CADR. Infiltrating lymphomononuclear cells induce epidermal aggression with a different degree of keratinocyte vacuolization or necrosis, whose intensity is somehow related with the severity of the reaction, and is expressed both in the acute skin eruption and in the positive patch test. The intensity of epidermal cytotoxicity in our skin samples, the predominance of $CD8^+$ T cells in the epidermis of some positive patch tests and the association of DRESS from allopurinol with a HLA class I, may further strengthen the contribution of $CD8^+$ T cells during the effector response in nonimmediate CADR.

In DRESS and other exanthema, the continuum in the intensity of the inflammatory infiltration and the epidermal aggression in skin biopsies, both in the acute reaction and in skin patch tests, suggests the existence of overlapping cases between DRESS and maculopapular exanthema with no systemic involvement, that is the object of an on-going study in our clinic.

The existence of common aspects between ACD and delayed CADR, explained at the introduction of this thesis, has been the backbone for conducting most of our studies in CADR and some of the work of this thesis. This relation between CADR and ACD has been reinforced by our work, by the similar behaviour of contact sensitizers and systemic drugs in *in vitro* tests and by the reproducibility of the histologic pattern of the CADR in the patch tests. This encourages us to go further using and ameliorating skin testing, particularly patch testing, in the diagnostic work-up of patients with delayed CADR and, also, in the study of pathomechanisms involved in the different patterns of CADR.

We must admit this is a very humble contribution as in Europe, and worldwide, there are at present several very active groups, with much more resources than ours, performing research within this field of drug hypersensitivity. We have occasionally shared our results with them and there are future plans to develop joint investigations.

We have, nevertheless, the advantage of evaluating our patients during the acute episode, which is time consuming and stressing in certain occasions, but allows us to appreciate better the clinical aspects of drug eruptions, understand their evolution and evaluate the overlap between the well defined classical phenotypes of delayed CADR.

Our hospital is reference for only 1 million inhabitants and there is no tradition to refer selected pathologies for special centres in Portugal. Therefore, the number of patients recruited yearly in our studies will hardly reach a very representative value, compared with those obtained in large cities or referral centres.

Although much is already known on pathophysiologic mechanisms in immune mediated CADR that can be relevant for our daily practice, many steps are still not understood and many questions have not found a proper answer.

Why, upon systemic exposure, in some instances the same drug induces a mild maculopapular exanthema, whereas in others it induces a multisystemic disease, involving only the mucosae or, sometimes, also the kidney or liver? The exclusive skin migratory capacity of some drug specific T cells may be the answer, but we do not know why some patients develop only these skin-prone migratory T cells, whereas there is no specific skin migration for others.

Does the drug skin binding capacity or its local metabolism dictate the preferential cutaneous involvement or the drug binding in other tissues is related to the associated systemic involvement?

How long and how tightly do drugs bind to skin cells? Can the cutaneous persistence of the drug justify the continued skin aggression, even after drug suspension?

Which cells are involved in antigen presentation in CADR? And do they influence the phenotype of CADR? And how?

Why does a maculopapular exanthema progress to DRESS or to SJS/TEN? Why is this progression is very slow, and sometimes seems to stop, whereas in other patients it progresses very quickly, occasionally reaching a maximum epidermal necrolysis in 24 to 48 hours in SJS/TEN?

Why, as in viral exanthema, generalized maculopapular CADR usually develop from head to toes?

Why do some CADR affect only some skin areas, as in the very limited round lesions in fixed drug eruption, or others have a particular distribution on the main body folds, as in AGEP or SDRIFE?

Why, even for CADR induced by the same drug and presenting with the same phenotype, the intensity of involvement is variable? Is it dependent of the dose of the drug or do individual patients' characteristics or concomitant factors (viral infection) matter?

Why, in patients with the same HLA genotype on exposure to the same drug, there is such wide risk variability, particularly between European/Japanese and Chinese populations?

Why for some drugs the risk associated with an HLA genotype is exclusively for one pattern of CADR, whereas for others it confers risk for all delayed reactions associated with that drug?

How do genetic factors, apart from the HLA genotype, dictate the initiation of the CADR and influence its evolution into distinct clinical patterns?

Which drug epitopes are really recognized by the immune system in each CADR? Do they vary among patients and patterns of CADR?

Why drugs like allopurinol never elicit positive patch tests?

Many more questions could be raised, but it is important to recognize that pathophysiologic mechanisms are progressively being unveiled giving us more tools to treat our patients and develop strategies to think cutaneous reactivity, not only in CADR, but also in other skin reactions that share inflammatory pathways with delayed CADR.

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