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***DIMENSIONS OF THE OLFACTORY BULB AND SULCUS AND THEIR
RELATION WITH OLFACTORY CORTICAL REGIONS IN USHER SYNDROME***

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ABSTRACT

BACKGROUND AND PURPOSE: Olfactory dysfunction is thought to be associated with Usher Syndrome (USH), although only few and controversial results are available. A recent animal study, along with other ciliopathies studies, provided support to the notion of olfaction underperformance in USH. We set out to report olfactory function for USH and both USH1 and USH2 genotypes. Olfactory bulb volumes, olfactory sulcus depths and olfaction-associated brain regions were also analysed.

MATERIALS AND METHODS: Twenty-six controls with no previous olfactory deficit were age-and-sex-matched to 32 USH patients (11 USH1, 21 USH2). Morphometric Magnetic Resonance Imaging (MRI) and a butanol threshold test were used to evaluate brain structures and olfactory function, respectively. OB volumes and OS depths were manually measured by three operators (JR, AP, SF) using Osirix, with excellent intraclass coefficient

tests. Averaged values across all measurements as well as brain regions' volumes as segmented by Freesurfer were used for statistical analysis in SPSS.

RESULTS: Olfactory thresholds were significantly higher in USH, $Z = 3.508$, $p = 0.000452$, and posthoc testing showed that this was mainly due to USH1 patients as compared to controls, $p = 0.000184$. OB volumes were not significantly different between groups, $F(1,52) = 0.034$, $p = 0.855$, and subgroups, $F(2,50) = 0.798$, $p = 0.456$. However, we did find butanol thresholds to be correlated with left OB volume for the USH1 subgroup alone ($r_s = -0.692$, $p = 0.018$). OS depths across groups were found to be significantly different as shown by repeated-measures ANOVA, $F(1,52) = 7.076$, $p = 0.01$. Analysis of subgroups revealed a significant decrease for left OS depth, $t(45) = 2.053$, $p = 0.047$, only for USH2 patients (adjusted mean = 5.415 mm, SD = 0.548 mm) versus controls (adjusted mean = 7.586 mm; SD = 0.492 mm).

As for brain regions, although differences were observed for a subgroup \times gender analysis, $F(2, 50) = 7.805$, $p = 0.001$, the overall model for both group and subgroup analysis were not significant, $F(1,52) = 1.980$, $p = 0.165$ and $F(2, 50) = 2.234$, $p = 0.118$, respectively.

CONCLUSIONS: The results provide evidence of olfactory dysfunction in patients with USH that correlates significantly with left OB volume specifically for the USH1 subgroup. Although olfaction is similar to controls, a decrease for left OS depth is present in the USH2 subgroup.

Keywords: Usher Syndrome, Smell, Olfaction Disorders

ABBREVIATIONS: USH – Usher Syndrome; USH1 – Usher syndrome type 1; USH2 – Usher Syndrome type 2; MRI – Magnetic Resonance Imaging; OB – olfactory bulb; OS – olfactory sulcus; OE – olfactory epithelium; RP – Retinitis Pigmentosa.

INTRODUCTION

First reported by Albrecht von Gräfe in 1858 and later named by Charles Usher in 1914,(1) Usher Syndrome (USH) is described as a heterogeneous and severely debilitating genetic disease of autosomal recessive nature, considered to be the most common cause of inherited deaf-blindness.(2,3) This disease has a worldwide prevalence of 3-8:100000(2,3) and of 9.7:100000 in Portugal.(Unpublished data)

Apart from being incurable, USH is known for its sensorineural hearing loss with progressive retinal degeneration in the form of Retinitis Pigmentosa (RP), featuring night blindness and loss of peripheral vision in early stages, with progression to complete blindness.(2,3) In fact, USH accounts for 18% of RP and 5% of inherited deafness cases.(3)

Additionally, USH may be subdivided in three types.(2–4) USH type 1 (USH1) is characterized by a congenital profound deafness associated with vestibular impairment and onset of RP during infancy. (2) USH type 2 (USH2) is the most common type and shows a milder hearing loss with no vestibular dysfunction and an onset of RP during puberty and early adulthood. In rare cases, a progressive loss of hearing along with variable expression of both vestibular dysfunction and RP onset lead to a diagnosis of USH type 3 (USH3). Those few cases with some atypical features have been described as atypical USH.(2,3,5)

Thirteen genes have been associated with USH so far,(2) most of them comprising functions related to cell adherence and protein scaffolding and signalling. This suggests proteins in these genes may participate in a multiprotein complex which is responsible for the development and maintenance of the hair bundles of the inner ear and similar structures. This may imply that multiple splice isoforms of USH genes play similar roles in different tissues, such as the outer segment and calyceal processes of photoreceptors in the retina, microvilli in the intestine, and the cilia in olfactory epithelium (OE).(3,5,6)

Due to the pathological mechanism of USH, olfactory involvement has long been suspected in this syndrome.(6–9) Although some studies have reported impairment of some aspects of this function,(7) others have contradicted these results.(8) Furthermore, recent animal studies have demonstrated that USH proteins are in fact expressed in the OE(6) renewing the interest in studying this in USH.

With the advent of MRI and other imaging methods, such as PET (Positron emission tomography) and fMRI (functional MRI), the study of olfaction has greatly evolved. Neural mechanisms of olfactory function can be studied not only in healthy subjects,(10–12) but also in Parkinson's disease (13,14) and schizophrenia,(15–17) among other pathologies with known olfactory deficits. For example, for some, as in Alzheimer's disease(18) and Bardet-Biedl syndrome (BBS),(19,20) olfactory bulb volume has been shown to be correlated with reduced olfaction, while in Parkinson's disease this has not been the case.(14) From this, interesting theories have emerged regarding the olfactory pathway and its function.(21)

We therefore hypothesize that as USH patients lack much of their sensory input, this group is expected to have significant differences regarding olfaction-related structures, similarly to other ciliopathies.(19,20) Also, studies have shown an atrophic pattern of the brain and cerebellum of USH patients.(22,23) Consequently, we aimed to evaluate the olfactory function in USH patients comparing with controls, focusing also on a subgroup analysis according to the USH genotype. In addition, we performed an assessment of the olfactory bulb volume and olfactory sulcus depth along with an analysis of different olfaction-associated brain regions' volumes in an attempt to elucidate this understudied feature of the disease.

MATERIALS AND METHODS

Thirty-two USH patients [20 male, 12 female; Mean age 46.59 with SD=13.54] were included in this study. Of these there were 11 USH1 and 21 USH2. Comparatively, 26 age-and-sex-matched controls [15 male, 11 female; Mean age 43.38 with SD=11.26] with no history olfactory impairment were also enrolled for this study. All of the pairings for subgroups (Control, USH1, USH2) were also age-and-sex-matched. Exclusion criteria for the study were missing data (n=2), previously documented olfactory impairment (n=1), abnormal neuroradiological assessment (n=1) and diagnosis of diabetes mellitus (n=1) as this disease is involved in atrophy of several brain regions,(24) even when accounted for body mass index.(25) Incorrect USH diagnosis (n=1) as detected by genetic testing was also an exclusion factor as well as a USH3 diagnosis (n=2) since a small subset presents challenges for the statistical analysis between subgroups.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Commission of Faculty of Medicine of University of Coimbra. Written informed consent was obtained from all participants after research procedures had been fully explained.

All USH patients were diagnosed and classified according to clinical criteria based mainly on ophthalmological and otolaryngeal examinations, with genotype being acquired on most subjects with a 90.2% mutated alleles detection rate.(26,27)

Both USH patients and controls were subjected to a thorough clinical examination and extensive review of clinical history in order to assess possible confounding variables for olfactory function tests and brain volumetric assessment.

Similarly to other studies,(8) a butanol olfactory threshold test was executed using a staircase procedure with a set of 8 solutions ranging from 4% to 0.002% following a 1:4

dilution with water as a solvent. Given this scale, the lower the concentration detected by the subject, the better the olfactory performance.(28–30)

Scanning was performed on a 3-Tesla scanner (Magnetom TrioTim, Siemens AG, Germany) at the Portuguese Brain Imaging Network, using a 12-channel birdcage head coil. Two T1-weighted ($1 \times 1 \times 1 \text{ mm}^3$ voxel size; Repetition Time (TR) 2.53 s; Echo Time (TE) 3.42 ms; Flip Angle (FA) 7° ; Field of View (FOV) $256 \times 256 \text{ mm}^2$; 176 slices) Magnetization-Prepared Rapid Acquisition with Gradient Echo sequences were acquired from each participant. For the OB and OS evaluation, scanning acquisition was based on previous studies(31) with one T2-weighted ($0.4 \times 0.4 \times 2 \text{ mm}^3$ voxel size; TR 6.4 s; TE 148 ms; FA 150° ; FOV $230 \times 230 \text{ mm}^2$; 30 slices) for each subject. All scans were reviewed by a neuroradiologist to assess possible brain injury or pathology.

Manual segmentation of the OB, moving distally from the change in diameter at the beginning of the olfactory tract,(14) and measurement of the length of the OS in the plane of the posterior tangent through the eyeballs (PPTE)(15,32) were done in Osirix v6.5.2 (Pixmeo S.A.R.L., Geneva, Switzerland) by three operators (JR, AP, SF). Since intraclass coefficient tests were of 0.928 [0.895 ; 0.953] and 0.962 [0.945 ; 0.976] for OB and OS, respectively, all measurements were averaged and used afterwards as such in all of the statistical analyses.

Freesurfer v5.3 software package (<http://surfer.nmr.mgh.harvard.edu/>) was used to perform cortical reconstruction and volumetric segmentation using an averaged T1-weighted MRI scan from all the subjects according to methodology previously described.(33)

Across all the analyses provided by this software, only a few areas for both hemispheres were selected according to the literature regarding olfaction.(12,16,34–36) They are as follows: amygdala, nucleus accumbens, orbitofrontal cortex (OFC), precuneus, parahippocampal region, insula and the entorhinal cortex. Due to an inability to segment the piriform cortex by Freesurfer v5.3, we included the temporal pole region as it is the

segmented area that most resembles the piriform cortex both at an anatomical and functional level.(37)

Statistical analyses was performed between groups (Control vs USH) and between subgroups (Control, USH1, USH2). As for MRI structural measures, OB and OS, we used repeated-measures analysis of variance (ANOVA) with the ‘between-subjects factor’ being either the group or subgroup along with gender. Side was the ‘within-subjects factor’. Age and estimated total intracranial volume were included as covariates. Regarding the butanol threshold, we performed Mann-Whitney and Kruskal-Wallis tests for group and subgroup analyses, respectively. Non-parametric Spearman correlations were then used to assess the correlation between butanol threshold and OB and OS. All analyses were performed using SPSS Statistics v23.0.0.2 (SPSS Inc., Chicago, IL, USA) with significance set at $p < 0.05$.

RESULTS

The statistical analysis showed that butanol thresholds do not correlate with age ($r_s = 0.210$, $p = 0.114$). Upon further inspection of this correlation by group, we find that this remains true for USH patients ($r_s = 0.015$, $p = 0.934$), regardless of the genotype (USH1: $r_s = 0.280$, $p = 0.405$; USH2: $r_s = 0.022$, $p = 0.925$). Importantly, age proved to be correlated with butanol threshold in healthy controls ($r_s = 0.487$, $p = 0.012$), suggesting that other disease related factors become more important than ageing.

Moreover, the butanol test thresholds proved to be significantly higher, $Z = 3.508$, $p = 0.000452$, in USH patients (Median = 0.14%) when compared with the control group (Median = 0.047%). Furthermore, a Kruskal-Wallis H ([Figure 1](#)) test showed a statistically significant difference between subgroups, $\chi^2(2) = 16.758$, $p = 0.00023$, emphasizing the difference (adjusted $p = 0.000184$) for controls versus USH1 (Median = 0.43%) but not for controls versus USH2 (Median = 0.047%) nor between USH1 and USH2.

In a repeated-measures ANOVA no overall effect in OB volume was found between groups, $F(1,52) = 0.034$, $p = 0.855$, and between subgroups, $F(2,50) = 0.798$, $p = 0.456$, and therefore no post hoc tests were performed. In addition, no effect of side was obtained for the model of OB volume, $F(1,52) = 0.008$, $p = 0.927$. Adjusted means for OB volumes are provided in Table 1.

As for olfactory sulcus between groups, we found a statistically significant main effect on overall OS depth, $F(1,52) = 7.076$, $p = 0.01$. Similarly to OB, OS depth was not found to be significantly different between sides for either group, $F(1,52) = 2.317$, $p = 0.134$. Adjusted means for OS are provided in Table 2.

However, after detecting an overall effect of OS only for the pairing Control vs USH2, $F(1,41) = 9.433$, $p = 0.004$, a statistically significant difference, $t(45) = 2.053$, $p =$

0.047, was found in post hoc tests for the left side with a decrease in USH2 patients (adjusted mean = 5.415 mm, SD = 0.548 mm) when compared to controls (adjusted mean = 7.586 mm; SD = 0.492 mm). The right side OS was not found to be significantly different, $t(45) = 1.938$, although a trend was present ($p = 0.060$).

Among both the bulbs and sulcus, butanol threshold was found to be negatively correlated only with the left OB volume ($r_s = -0.279$, $p = 0.034$). Follow-up analysis showed that in addition of being limited to USH patients ($r_s = -0.393$, $p = 0.026$), this was further limited to the USH1 subgroup ($r_s = -0.692$, $p = 0.018$).

As for brain regions, we found no overall effect on all the brain areas referred to previously, $F(1,52) = 1.980$, $p = 0.165$, and therefore did not run any post hoc tests. On a subgroup analysis, gender was found to have an effect in brain regions analysis, $F(1, 50) = 9.712$, $p = 0.003$, although USH did not, $F(2, 50) = 2.234$, $p = 0.118$. We therefore combined USH and gender in the same model, which was statistically significant, $F(2, 50) = 7.805$, $p = 0.001$. Therefore, when analysing the data split by gender we found that for groups, males had a non-significant model, $F(2,30) = 2.285$, $p = 0.119$, while for females the opposite was the case, $F(2,18) = 10.501$, $p = 0.001$. Post-hoc test results for the significant areas in the significant pairwise comparisons are illustrated in Tables 3, 4.

Upon these findings, a correlation between butanol threshold between OFC and precuneus volumes on both sides was performed, with only the right OFC showing a statistically significant correlation ($r_s = -0.310$, $p = 0.018$). When separated by group, this effect is lost for both controls ($r_s = -0.242$, $p = 0.234$) and USH ($r_s = 0.282$, $p = 0.118$) subjects.

DISCUSSION

This study sheds new light on olfactory function in USH patients, an area we believe to be surprisingly understudied and a controversial topic.(7,8)

The results show reduced olfaction in USH patients, mainly in the USH1 subgroup with USH2 performing similar to controls. Although contradicting results reported by Seeliger *et. al* concerning butanol threshold measurements (8) and Zrada *et. al* with the phenylethyl alcohol odour test, a University of Pennsylvania Smell Identification Test (UPSIT) on 22 USH patients by the latter corroborates our findings.(7) This is further confirmed in a study on another ciliopathy, Bardet- Biedl Syndrome (BBS), that found decreased olfactory function across all patients.(19)

Put into perspective, this supports Jansen *et. al* findings of the presence of USH proteins in the olfactory epithelium and their interaction with olfactory signalling proteins as demonstrated in mice models of USH.(6) Therefore, this disease seems to affect the development of stereocilia and stereocilia-like structures in not only the inner ear and in the retina, but also in the nasal cilia. Anecdotally, it was Arden *et. al* in 1979 that appears to have been the first to describe abnormalities in the nasal cilia of patients with this pathology in a study of 11 RP patients, of which 6 were USH.

Moreover, different genotypes, and therefore different mutations, seem to have a different impact in this regard. Although USH2 was not included in air-phase electro-olfactogram recordings in the mice model for USH in Jansen *et. al* tests, in humans this subgroup seems to perform similarly to controls as seen in our data. This suggests that USH1 proteins' isoforms in the OE might play a more dominant role in comparison to USH2's in the multiprotein complex. Proven to be true, this could explain the absent deficit regarding

olfaction for the latter and the significant reduction for the former. Further animal and clinical studies may provide additional insight towards this matter.

Furthermore, as reported in healthy subjects, olfactory performance showed a significant negative correlation with age in our control group.(21,36,38) This has been theorized to be due to repeated viral infections of the upper airway, decrease in neurotransmitter, and degeneration of OE, among other things.(36) However, the same trend was found for USH patients, though this was not statistically significant, suggesting that other pathologic factors might become more relevant.

As pointed out by several studies,(11,21) the OB is considered to be the primary place for processing olfactory information, making the connection between the peripheral and central nervous system via the olfactory tract. Also, OB volume has been found to be correlated with olfactory performance in healthy subjects(10,11,17,21,36,39) as well as for several diseases.(18,40,41) Similarly to by Braun *et. al* findings in BBS patients,(20) we also found a correlation between olfactory performance and left OB volume for USH1, with OB volume by itself not being significantly different for either hemisphere between groups and subgroups. As for OS depth, we found no correlation at all.

Given the link for the butanol test and OB volumes and OS depth, contrary to what would be expected, these appear to remain unchanged in the USH group. This may be explained by a possible effect of neuroplasticity of the OB(11,17) as a result of compensation for early loss of vision and hearing, as described by Rombaux *et. al* in early blind subjects.(42) Unfortunately, since our study only provides a single point in time for volumetric assessment of the various structures we cannot assess this interaction and further studies are necessary to elucidate this matter. Interestingly, the correlation found between olfaction and left OB volume for USH patients may suggest that any possible interaction may only happen for the left side. This finding has to be reconciled with the notion of a right-side

dominance for olfaction in OB, OS and brain regions.(41,43,44) Nevertheless, the finding of a decrease in left OS depth in USH2 *versus* the control groups further supports our pattern of results.

Several brain regions were found to be functionally associated for olfaction.(12,16,34–36) In cases such as idiopathic Parkinson’s disease, it is hypothesized that if the patients present with olfactory dysfunction along with a similar OB volume as controls, the problem would lie in only higher-level cortical areas for processing of olfaction.(14,43) This would most likely imply a process of atrophy for the selected brain regions, but not for the OB, which did not happen to be the case in our study either. However in USH one would expect more damage in the regions closer to the loss of afferent input. Conclusions from brain regions analysis however, become quite challenging due to the heterogeneity of USH and, for example, due to psychotic symptoms that may develop in USH patients but not in related diseases.(45,46) These symptoms may alter brain function also at a higher level and possibly lead to higher level pathological development than what would be expected by visual, hearing, and olfaction impairment alone.

We suggest that olfactory threshold testing should be regularly included in the clinical assessment for USH patients. Also, we did not test other functions of olfaction, such as olfactory discrimination and identification, which could be useful in providing better evidence to support an olfactory deficit in these patients. This can be done using the UPSIT test or a full TDI (Threshold, Discrimination, Identification) score.

Upon future replication studies, if consistent with our findings, we further advise to consider olfactory testing as a differentiation factor for the diagnosis between USH1 and USH2 if genetic analysis is unavailable. USH3 was not included in our analysis due to our small sample of this subset (n=2), although investigation in this group with significant sample size, could provide further insight to the spectrum of olfactory function across USH. This

could be achieved in the Finnish population or the Ashkenazi Jews, since a higher rate of USH3 has been reported in these groups.(3)

CONCLUSION

We conclude that there appears to exist a significant olfactory dysfunction for USH1 patients, but not for USH2, which only correlated with left OB volume for the first subgroup. However, both OB and OS measurements were not found to be significantly different for groups or USH subgroups, raising relevant questions on the effects of the lessened olfactory performance in the olfactory pathway and upstream brain structures. Apart from the effects found within the female group, the fact that no volumetric assessment of the brain regions was significantly different when accounting for both genders, suggests the need for future studies. Altered nasal cilia may also be associated with a plasticity for the OB which may add to the large clinical heterogeneity of USH. Further molecular studies on the nasal cilia of USH patients should help further understand such heterogeneity. In addition, anatomical studies much like our own may also benefit from further studies with functional methodologies based on PET and fMRI.

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ANNEXES

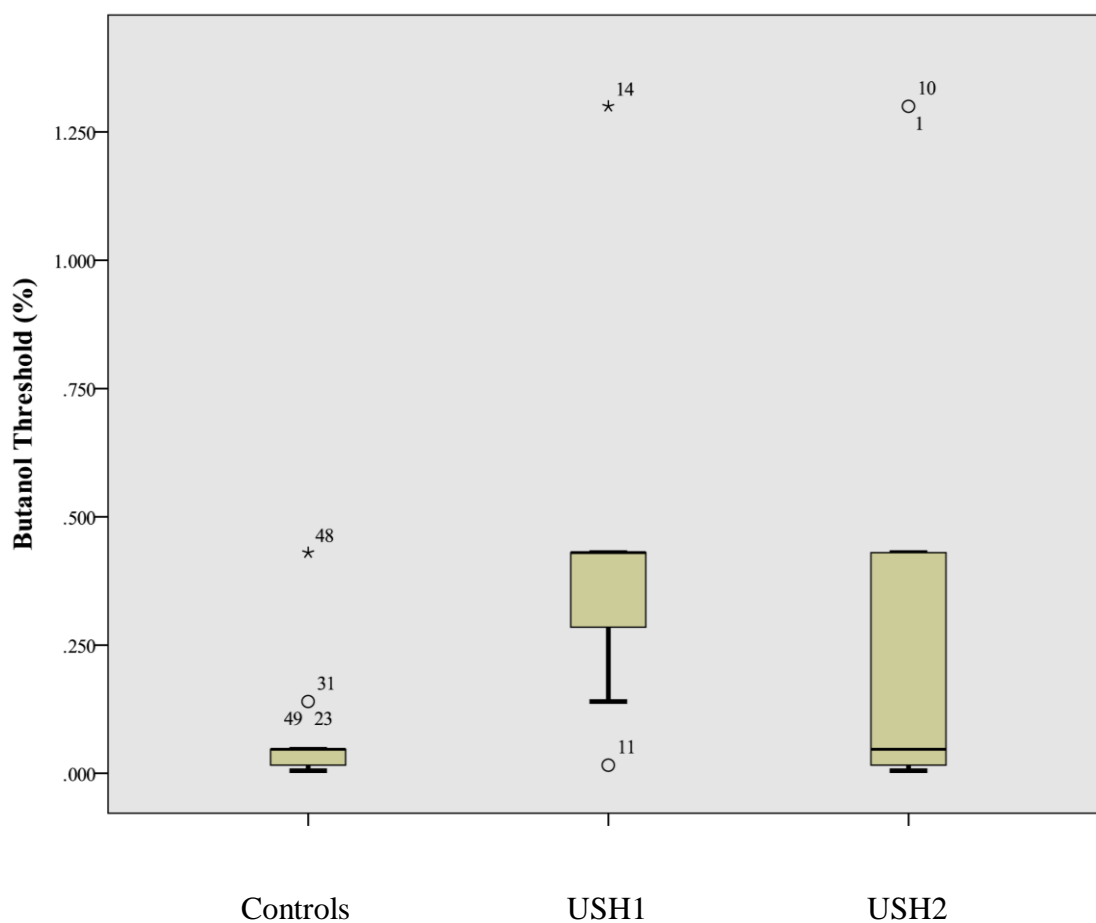


Figure 1 - Kruskal-Wallis H test, $\chi^2(2) = 16.758$, $p = 0.00023$, for a subgroup analysis for butanol threshold test.

Table 1 - Olfactory bulbs volume for Control and USH groups. ROB – Right Olfactory Bulb. LOB – Left Olfactory Bulb.

		ADJUSTED MEAN (mm ³)	SD (mm ³)
Control	ROB	56.74	2.85
	LOB	57.53	2.57
USH	ROB	57.60	2.68
	LOB	55.38	2.42

Table 2 - Olfactory sulcus depths for Control and USH groups. ROS – Right Olfactory Sulcus. LOS – Left Olfactory Sulcus.

		ADJUSTED MEAN (mm)	SD (mm)
Control	ROS	7.83	0.55
	LOS	7.44	0.49
USH	ROS	5.91	0.52
	LOS	5.82	0.47

Table 3 - Statistically significant areas for the female gender, between Control and USH1 subgroups.

	<i>t</i>	df	<i>p</i>	Control		USH1	
				Adjusted mean (mm ³)	SD (mm ³)	Adjusted mean (mm ³)	SD (mm ³)
LEFT OFC	3.387	35	0.007	11217.68	245.4	9480.84	453.47
LEFT PRECUNEUS	4.395	35	0.001	8470.38	148.36	7050.26	285.79
RIGHT OFC	3.218	35	0.009	10969.51	174.29	9747.78	335.75
RIGH PRECUNEUS	3.893	35	0.003	8867.56	160.41	7507.29	309.2

Table 4 - Statistically significant areas for the female gender, between USH1 and USH2 subgroups.

	<i>t</i>	df	<i>p</i>	USH1		USH2	
				Adjusted mean (mm ³)	SD (mm ³)	Adjusted mean (mm ³)	SD (mm ³)
LEFT OFC	-4.686	30	0.002	9291.59	343.61	11199.03	187.70
LEFT PRECUNEUS	-2.653	30	0.029	7060.83	464.44	8520.17	253.70
LEFT TEMPORAL POLE	-3.164	30	0.013	1905.51	114.91	2336.16	62.77
RIGHT OFC	-2.569	30	0.033	9760.05	349.30	10823.21	190.80
RIGH PRECUNEUS	- 2.328	30	0.048	7299.37	455.79	8556.10	248.98