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Salivary proteome and microbiome in pregnancy and postpartum: An exploratory study on the relation with arterial hypertension

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ABSTRACT

Objectives: Arterial hypertension (AH) influences salivary gland physiology and oral health, being associated with a higher incidence of periodontal disease in pregnant women. Evidence points to a bidirectional relationship between the oral microbiota and blood pressure regulation. Therefore, this study aimed to characterize the oral health of pregnant women and AH-associated changes in the salivary proteome and microbiome during pregnancy and postpartum.

Design: Ten healthy women and ten women with AH were enrolled. Saliva was collected during pregnancy and six months postpartum. The salivary proteome was characterized by shotgun label-free mass spectrometry analysis. Specific proteins were validated through parallel reaction monitoring (PRM). The oral microbiota was characterized via 16S rRNA gene amplicon sequencing (V4 region). The periodontal health and the caries history was assessed during pregnancy.

Results: Pregnant women with AH had lower junction plakoglobin (JUP)- and desmoplakin (DSP)-specific peptide levels than healthy women, confirmed by the PRM approach. The levels of these proteins correlated negatively with periodontal health indexes, which were higher in pregnant women with AH. In AH, nitrate-reducing microorganisms had lower abundance, correlating positively with JUP and DSP-specific peptides.

Conclusions: The salivary proteome and microbiota are shaped by AH during and after pregnancy. Further research is required to understand the underlying mechanisms impairing oral health in AH.

1. Introduction

During pregnancy, the female body undergoes severe hormonal, metabolic, and immunological changes (Gursoy et al., 2016). In particular, changes in the oral ecosystem predispose pregnant women to an exacerbated gingival inflammation, which is considered the most common oral manifestation during pregnancy and a potential risk factor for developing oral infections and adverse pregnancy outcomes (Goncalves Lda et al., 2011). The underpinning mechanism seems to be a progressive increase of estrogen and progesterone, which peak at the end of the 3rd trimester of pregnancy and have a particular role in gingival and salivary physiology (Amit Kumar Dey, Kumar, Singh, Ranjan, Thiruvengadam, Desiraju, Kshetrapal, Wadhwa, Bhatnagar, Rashid, Malakar, Salunke, Maiti, Das, et al., 2020; Lasisi & Abdus-Salam, 2018).

Pregnant women with arterial hypertension (AH), an increasingly

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prevalent condition worldwide and associated with a panoply of pregnancy complications, tend to have a higher incidence of periodontal disease compared to healthy pregnant women (Pralhad et al., 2013). This interplay may influence salivary gland physiology. Some studies with animal and human subjects observed impaired salivary gland pathways and function (e.g., Ca^{2+}/AQP5 pathway) and a higher concentration of carbohydrates and proteins in AH subjects (Mohiti et al., 2020; Nazeer et al., 2017; Zhang et al., 2017). Furthermore, evidence is mounting on a bidirectional relationship between the oral microbiota and cardiovascular health, namely with blood pressure regulation (Li et al., 2022). For instance, LaMonte et al. (2022) showed that AH was associated with a higher relative abundance of the genera *Veillonella*, *Oribacterium*, and *Prevotella*, and a lower relative abundance of *Leptotrichia*, *Streptococcus*, and *Rothia*. One possible mechanism is related to nitric oxide (NO) homeostasis. NO is a potent vasodilator and blood pressure modulator (Duncan et al., 1995). Oral commensal bacteria contribute to the production of this molecule via the entero-salivary pathway by reducing dietary nitrate in nitrite, a precursor of NO (Raizada et al., 2017).

The salivary proteome includes all proteins synthesized in the salivary glands or proteins from other body locations that reach the oral cavity by crossing the salivary gland endothelium and epithelium (Saitou et al., 2020). There are 6 structurally related major classes: histatins, proline-rich peptides (acid, basic and neutral), statherins, and cystatins (Schulz et al., 2012). Salivary proteins released in the oral cavity undergo several modifications within salivary ducts, such as proteolytic cleavage, partial deglycosylation, and protein-protein complex formation (Helmerhorst & Oppenheim, 2007). The salivary low-molecular-weight (LMW) proteome represents a group of small proteins and peptides that constitute a considerable part of saliva (Morzel et al., 2012). These peptides can be from oral origin (e.g., major and minor salivary glands, gingival crevicular fluid) but also from plasma, serum, and microbial origin (Amado et al., 2010; Rabe et al., 2019). The salivary LMW proteome is particularly appealing for research since it is composed of bioactive peptides and protein fragments, which may reflect important changes in oral proteolytic activity (Morzel et al., 2012; Zhu et al., 2020). For these reasons, the salivary proteome is not only an essential part of the defense mechanisms in the oral cavity but also represents a unique diagnostic fluid to detect biomarkers of physiological changes both orally and systemically (Hu et al., 2007). During pregnancy, salivary proteomics has been mostly applied in an attempt to discover potential biomarkers to explore the association with pregnancy-related complications, such as early pregnancy loss or preterm delivery (Balan et al., 2021; A. K. Dey, Kumar, Singh, Ranjan, Thiruvengadam, Desiraju, Kshetrapal, Wadhwa, Bhatnagar, Rashid, Malakar, Salunke, Maiti, Das, et al., 2020). However, it remains to be elucidated whether AH also shapes the salivary proteome of women from pregnancy to postpartum and if it is associated with oral health and microbiota.

Therefore, this study aims to explore the oral health status, salivary proteome, and the oral microbiota of a pregnant population to i) characterize the oral health of a pregnant women population, including healthy and hypertensive women; ii) identify the AH-associated changes in the salivary proteome and microbiota, both in pregnancy and 6 months after delivery; iii) explore the existence of correlations between salivary proteome and oral microbiome in pregnancy.

2. Material and methods

2.1. Recruitment of participants

The project was approved by the ethics committee of *Centro Hospitalar Universitário de São João*, Portugal (CHUSJ) in December 2018 (Nº294/2018) and by *Unidade Local de Saúde de Matosinhos*, Portugal (ULSM) (86/CE/JAS), with authorization for the reuse of Hospital Clinical Records for Research. A total of 20 participants (10 healthy and 10 arterial hypertensive (AH)) were recruited at the Obstetrics Service of CHUSJ and ULSM. Arterial hypertension was defined as systolic blood pressure 140 mmHg and/or diastolic blood pressure 90 mmHg measured in the office or in-hospital before 20 weeks of gestation (Regitz-Zagrosek et al., 2018). Exclusion criteria were age below 18 years, twin pregnancies, pre-existing cardiomyopathy, chronic obstructive airway disease, active systemic infection, type 1 Diabetes Mellitus and genetic syndromes. Written informed consent was obtained from all participants.

2.2. Data and sample collection

The participants were clinically characterized regarding maternal health history, including history of systemic diseases, medication intake, and blood pressure. Smoking, drinking, nutritional, and oral hygiene habits were registered. A complete oral examination was performed in the 3rd trimester of pregnancy on all AH-women (n = 10) and most healthy women (n = 7). Three healthy pregnant participants did not have an oral health screening due to COVID-19 restrictions. Moreover, the initially planned follow-up clinical evaluation 6 months after delivery was not possible for the same reason. The caries history was characterized via decayed, missing, and filled surface (DMFS) index, and periodontal health - via clinical attachment loss (CAL), bleeding on probing (BOP), and plaque index (PI). Participants donated unstimulated saliva in the 3rd trimester of pregnancy and 24–28 weeks after delivery. Approximately 5 mL of unstimulated saliva samples were collected into a sterile 50 mL tube at room temperature and were immediately placed on ice. The participants were asked to collect the sample at least 1 h after a meal or toothbrushing. After collection, samples were aliquoted in four 1.5 mL microtubes (1 mL per microtube) and immediately stored at −80°C until further processing.

2.2.1. Proteomic analysis

2.2.1.1. Sample processing. One aliquot (1 mL) of each participant's saliva sample was used for proteomic analysis. All samples were thawed and processed on the same day. Samples were centrifuged at 12,000 ×g for 15 min at 20°C to remove possible debris, such as cellular debris, insoluble material, and food remnants. Protein concentration was measured using a commercial BCA assay (Pierce, ThermoFisher, Rockford, IL, USA). The volume equivalent to 300 μg of protein was separated and treated with a protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail). From this, 25 μg of protein from each sample was reserved for whole proteome analysis. A total of 250 μg of protein from each sample was then filtered with a 30 kDa spin filter (Vivaspin® 500, Sartorius Stedim Lab Ltd, United Kingdom) by centrifugation at 12,000 ×g for 10 min at 20°C to obtain the low molecular weight (LMW) proteome. Two extra centrifugations were performed to maximize peptide recovery: one after resuspending the retentate with 200 μL of 50 mM NH_4HCO_3 and another with 0.1 % SDS. The resulting filtrates were pooled and frozen at −80°C until proteome and peptidome analysis.

2.2.1.2. Shotgun Label-free quantitative proteomic analysis. For the whole proteome analysis, 20 μg of proteins from the processed samples were used. The total volume of each sample was adjusted to 40 μL with 100 mM triethylammonium bicarbonate buffer (TEAB). The proteins were reduced and alkylated with tris(2-carboxyethyl)phosphine (TCEP) (10 mM) and 2-chloroacetamide (CAA) (50 mM) followed by incubation at 95°C for 5 min. Then proteins were cleaned up using 100 μg of SpeedBeads magnetic carboxylate-modified particles mixed in a ratio of 1:1 (GE45152105050250 and GE65152105050250, GE Healthcare) (Hughes et al., 2014). Digestion was performed by adding trypsin in a 1:20 ratio at 37°C overnight and terminated by adding trifluoroacetic acid (TFA) to a final concentration of 0.5 %.

The Evosep One (Evosep) HPLC system coupled with timsTOF SCP

(Bruker Daltonics) was used for LC-MS/MS. Prior to the analysis, the processed protein samples were loaded onto Evotip Pure™ (Evosep). Peptides were separated using the 60 samples/day method and a reverse-phase analytical column (Dr. Maisch C18 AQ, 3 μm beads, 100 μm ID, 8 cm long, Evosep), heated to 60°C. Two mobile phases were utilized: phase A consisted of 2 % acetonitrile (ACN) and 0.1 % formic acid (FA), while phase B contained 98 % ACN and 0.1 % FA. MS spectra were acquired and recorded within the 100–1700 *m/z* range, while the ion mobility was scanned from 0.6 to 1.6 Vs/cm^2. The method consisted of a TIMS survey scan lasting 166 ms, followed by 10 Parallel Accumulation Serial Fragmentation (PASEF) MS/MS scans. The total cycle time for this process was 1.9 s. The target intensity was set at 20,000 with an intensity threshold of 1000. Precursors for data-dependent acquisition were fragmented with an ion mobility-dependent collision energy that increased linearly from 20 to 59 eV.

2.2.1.3. Targeted proteomic approach. The list of peptides for parallel reaction monitoring (PRM) and acquisition parameters were prepared using Skyline software (Supplementary Table 1) (MacLean et al., 2010). For Parallel Reaction Monitoring (PRM) analysis, the processed protein samples were desalted using the StageTip approach. LC-MS/MS analysis was done using Vanquish Neo UHPLC system (Thermo Fisher) coupled with timsTOF SCP (Bruker Daltonics). The processed samples were loaded into a reverse-phase trap column (Pepmap Neo C18 5 μm 0.3 ×5 mm, ThermoScientific) and subsequently into a reverse-phase analytical column (PepSep C18, 0.15 × 150 mm, Bruker Daltonics). Both columns were heated to 60°C. Mobile phases A and B consisted of 2 % acetonitrile (ACN)/0.1 % formic acid (FA) and 98 % ACN/0.1 % FA, respectively. Peptides were separated using a liner gradient over 14 min, starting from 1 % to 35 % solvent B. Parameters of MS acquisition method were as described in Supplementary table 1. The time-scheduled acquisition boxes were set with 4 min of tolerance on retention time calculated by Skyline software.

2.2.1.4. Proteome analysis. Proteins were identified using MaxQuant software (version 1.6.17) (Tyanova et al., 2016) and peak lists were searched with the Andromeda search engine (Cox et al., 2011) against the *Homo sapiens* database downloaded from Uniprot (https://www.uniprot.org). Parameters used for the database search were as follows: enzyme specificity: trypsin, allowing up to two missed cleavages; fixed modifications: carbamidomethylation of cysteine; variable modifications: N-terminal protein acetylation and methionine oxidation. Precursor ion tolerance was set at 20 ppm, whereas the mass tolerance for MS/MS fragment ions was set at 0.5 Da. PSM (Peptide Spectrum Match) and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1 %. Label-Free Quantification (LFQ) of proteins was done using the MaxLFQ algorithm integrated into MaxQuant (Cox et al., 2014); the minimum ratio count was set to 2. Then data were analyzed using Perseus software (version 1.6.15.0) (Tyanova & Cox, 2018). The data were filtered to eliminate hits to the reverse database, contaminants and proteins only identified with modified peptides. LFQ intensity values were transformed by log2 and normalized using Z-score. Statistical analyses were performed with algorithms integrated into Perseus. The STRING database (version 11.5; (Szklarczyk et al., 2018)) was sourced (accession on May 31, 2023) to uncover interactions between dysregulated proteins as well as for functional enrichment analysis (using the Gene Ontology add-on). Only interactions with, at least, medium confidence (STRING score ≥0.4) and significantly enriched biological processes were considered.

2.2.2. Microbiota analysis

2.2.2.1. DNA isolation. Another aliquot (1 mL) of saliva from each participant was used for microbiota analysis. DNA was isolated from saliva in one batch. To control for potential contaminations, blank isolation controls and PCR controls (reactions without template DNA), were added as negative controls. In addition, experimental controls, such as RNA/DNA-free 1.5 mL microtubes, were included. The vials were thawed, vortexed, and 200 μL of the saliva was transferred to an assigned well. Each 1.1 mL deep-well plate contained 250 μL 0.1-mm Zirconia beads, 200 μL of phenol (Rotiphenol, Carl Roth GMBH&Co. KG, Germany) and 200 μL of lysis buffer (MagMini DNA isolation kit, LGC Genomics Ltd, UK). The deep well plate was sealed with a silicone lid and placed in a Mini-BeadBeater-96 (BioSpec Products, Bartlesville, OK, USA) for 2 min, 4 times, at 2100 oscillations/min. DNA was extracted and purified using the MagMini DNA Isolation Kit (MagMini DNA isolation kit, LGC Genomics Ltd, UK). Bacterial DNA concentration in the samples after purification was determined by qPCR, with universal primers specific to the bacterial 16S rRNA gene.

2.2.2.2. PCR amplification and sequencing. The V4 hypervariable region of the 16S rRNA gene was amplified with barcoded forward and reverse primers, using 1 ng DNA with 1 μM of each primer and performing 30 amplification cycles [28]. Amplicons were pooled equimolarly; PCR products of isolation blanks, sample blanks, microtubes, and negative PCRs (PCR mix with DNA-free water) were included. Paired-end sequencing of the DNA was conducted on the MiSeq platform (Illumina, San Diego, CA, USA) in 1 run, using a V3 kit and 2 × 251 nucleotides (TNO, Zeist, the Netherlands). The flow cell was loaded with 7 pmol DNA containing 30 % PhiX.

2.2.2.3. Data processing. All sequences were processed together. The reads were merged, quality-filtered and checked for possibly remaining PhiX reads as previously described (Koopman et al., 2016) with the following exception: a maximum of 25 mismatches (10 %) was used in the overlap region during read merging. Next, the quality-filtered sequences were denoised using UNOISE3 (usearch v10.0.240, 32-bit (Edgar, 2016)). Mapping of the sequences to the "zero-radius OTUs" (zOTUs) was carried out using usearch_global with –maxaccepts 128 –maxrejects 1024 –maxhits 1, for higher sensitivity during mapping. The representative most abundant zOTU sequences were assigned a taxonomy as previously described (Koopman et al., 2016), however, a trimmed version of HOMD v14.51 (Chen et al., 2010) was used as taxonomic reference database for the RDP naïve Bayesian classifier with a minimum confidence of 80 % (Wang et al., 2007). The zOTU-table was filter for at least 1000 reads per sample, using R version 4.2.0 ("R Core Team 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.") and the phyloseq package v1.40.0 (McMurdie & Holmes, 2013), in order to allow comparisons among different samples. For calculating the alpha diversity indexes, the zOTU-table was subsampled at a depth of 7000 reads per sample.

2.2.2.4. Microbiota bioinformatic analysis. The Shannon diversity index (alpha diversity) for each group is presented as median [minimum; maximum]. The Shannon diversity index was assessed between the two groups by performing Mann-Whitney U tests. For multivariate analyses of microbial profile data and ordination of the samples, the zOTU-table was centered log-ratio (clr) transformed and ordinated using Principal Component Analysis (PCA). Differences in microbial profiles among the study groups (beta diversity) were assessed with one-way Permutational Multivariate Analysis of Variance (PERMANOVA, 9999 permutations; vegan v2.6–2 (Oksanen et al., 2022)) using the Aitchison distance. These analyses were also performed using R ("R Core Team 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.") and phyloseq (McMurdie & Holmes, 2013). The assessment of differentially abundant genera for the paired and unpaired groups was performed on non-subsampled data. First, taxa was aggregated with a 10 % prevalence filter on genus-level (Nearing et al., 2022). Then, data

was analyzed using ANOVA-Like Differential Gene Expression Analysis (ALDEx2; R package version 1.28.1; (Fernandes et al., 2014)) in R (aldex.ttest; paired.test=F). The minimum significance level after Benjamini-Hochberg correction was 5 %, as suggested by Fernandes et al. (2013).

2.2.2.5. Statistical analysis of the sociodemographic and clinical data. Univariate analyses of the sociodemographic and clinical data were performed using SPSS Statistics version 28 (IBM). The categorical variables were described through absolute or relative frequencies (%) and analyzed using Pearson's chi-square test or Fisher's exact test when more than 1 cell displayed expected counts less than 5. Continuous variables were described using median [range] and analyzed with Mann-Whitney *U* test for independent samples. To correlate data from the microbiome and proteome, the raw zOTU-table and LFQ intensity values were log-2 transformed and Spearman correlations were applied.

3. Results

3.1. Clinical and demographic characterization

This prospective study followed a group of healthy (n = 10) and arterial hypertensive (AH) (n = 10) women in the 3rd trimester of pregnancy (32 [29; 35] weeks of gestation) and six months after delivery (6 [4; 9] months after delivery). Table 1 displays the clinical and demographic characterization of the study groups. No differences between groups were found by age and body mass index. The AH group had a higher percentage of women taking acetylsalicylic acid and heparin in the 3rd trimester of pregnancy.

Regarding the blood pressure measurements, women with AH scored significantly higher. We did not observe differences in oral hygiene habits between groups in the 3rd trimester and 6 months after delivery. However, significant differences were observed between the groups by DMFS, percentage of sites with a CAL > 3 mm, BOP, and PI during the 3rd trimester, with participants from the AH group scoring higher in all clinical parameters.

Table 1
Demographic and clinical characterization of the study groups. Results are shown as relative frequency (%) or median [range].

	Healthy (n = 10)	Arterial Hypertension (n = 10)
Systolic Blood Pressure		
3rd trimester (mmHg)	120 [102; 120]	120 [100; 174]
6 months after delivery (mmHg)	110 [102; 130]	120 [110; 140]$^{\#}$
Diastolic Blood Pressure		
3rd trimester (mmHg)	60 [60; 80]	80 [50; 105]$^{\#}$
6 months after delivery (mmHg)	70 [60; 80]	80 [80; 100]$^{\#}$
Age (in years)	34.4 [26.3; 37.3]	37.6 [32.1; 41.7]
Body mass index before pregnancy	26.5 [18.7; 29.4]	27.2 [22.3; 30.9]
Maternal obesity (%)	0 %	30 %
Smokers (%)		
3rd trimester of pregnancy	20 %	20 %
6 months after delivery	11.1 %	12.5 %
Medication (3rd trimester) (%)		
Antihypertensive	0 %	40 %
Acetylsalicylic acid /heparin	20 %	100 %*
Anti-depressants	0 %	10 %
Anxiolytics	0 %	10 %
Medication (6 months after delivery) (%)		
Antihypertensive	0 %	30 %
Anti-depressants	0 %	10 %
Oral health habits – 3rd trimester		
Dental appointments (n/last year)	2 [0; 3]	2 [0; 6]
Frequency of toothbrushing (n/day)	2 [1; 3]	2 [1; 2]
Oral health habits –6 months after delivery		
Dental appointments (n/last year)	3 [0; 12]	1 [0; 10]
Frequency of toothbrushing (n/day)	3 [1.0; 6]	2 [1.0; 3]
Oral Health 3rd trimester^{+}		
DMFS	5 [2; 28]	25 [3; 56]$^{\#}$
% of sites with CAL> 3 mm	0.6 [0.0; 11.7] %	3.7 [0.7; 21.7] %$^{\#}$
BOP (%)	8.3 [4.2; 12.4] %	23.0 [11.3; 54.7] %$^{\#}$
PI (%)	67.9 [50.0; 75.9] %	88.3 [56.7; 98.0] %$^{\#}$

* $p < 0.05$; Fisher's Exact test. $^{\#}p < 0.05$; Mann-Whitney *U* test. $^{+}$ Healthy (n = 7) and arterial hypertension (n = 10).

3.2. The association of arterial hypertension with the salivary proteome

In order to assess if the oral proteome is related to AH during pregnancy, we started by comparing the whole salivary proteome between pregnant women with and without AH. We also compared the proteome at postpartum to evaluate if the observed AH-associated changes were specific to the pregnant condition. Overall, 257 proteins were identified in the samples collected during pregnancy, and 255 proteins were identified in the samples collected six months after delivery. Zymogen granule protein 16B (ZG16B) was found to be lower (fold-change=-1.31; p = 0.026) in pregnant women with AH. Curiously, these differences were not observed in postpartum. Indeed, only one protein was down-regulated (fold-change=-1.33; p = 0.034) in postpartum in AH women (cocaine esterase(CES2)).

Aiming to gain sensitivity in the analysis and considering the relevance of proteolytic activity in saliva resulting in protein degradation, we then focused on the analysis of the LMW proteome. Therefore, we enriched this fraction and followed a similar shotgun workflow. With this approach, 99 salivary proteins were identified in the 3rd trimester of pregnancy. From these, we found small proline-rich protein 3 (SPRR3, fold-change=-3.77; p = 0.0060), fragments of desmoplakin (DSP, fold-change=-1.71; p = 0.028) and junction plakoglobin (JUP, fold-change=-1.27; p = 0.027) to be significantly reduced in AH women saliva (Fig. 1 A). Protein-protein interaction analysis using STRING depicted an interaction between DSP and JUP (Fig. 1B). Functional enrichment analysis in the same tool uncovered the biological processes in which the dysregulated proteins take part (Supplementary Table 2).

We searched for potential correlations between these proteins (Fig. 1B) and oral health parameters, given the differences in periodontal health between these two groups (Table 2). Interestingly, JUP and DSP correlated negatively with the number of sites with significant clinical attachment loss (>3 mm). JUP also correlated negatively with BOP.

A total of 92 proteins were identified in the LMW saliva fraction collected 6 months after delivery. Galectin-7 (LGALS7) was significantly reduced in the saliva of women with AH (fold-change=2.12; p = 0.0076). This protein is involved in the regulation of cell-cell and cell-matrix interactions and also has a pro-apoptotic role. Lamin-B1-related peptides (LMNB1) were borderline decreased in the same group (fold-change=-1.62; p = 0.049). In contrast, the fragments of some large proteins were found in significantly higher levels in the saliva of pregnant women with AH, including complement proteins (C3, C4A, C4B) or immunoglobulin components (such as IGKC, IGKV3-D20), suggesting a higher level of proteolysis in the saliva of AH women after delivery (Fig. 2).

3.3. Validation by parallel reaction monitoring

Given the results observed with the LMW proteome fraction and the correlations of the fragments of proteins JUP and DSP with oral health parameters, the peptides of these proteins were selected for validation by parallel reaction monitoring.

We observed similar trends for the peptides in the targeted MS approach for both DSP and JUP. All PRM-validated peptides from the selected proteins were reduced in the AH participants (Fig. 3), but only two DSP peptides reached statistical significance (AELIVQPELK and YGDGIQLTR). Nevertheless, all JUP peptides and all except one of the

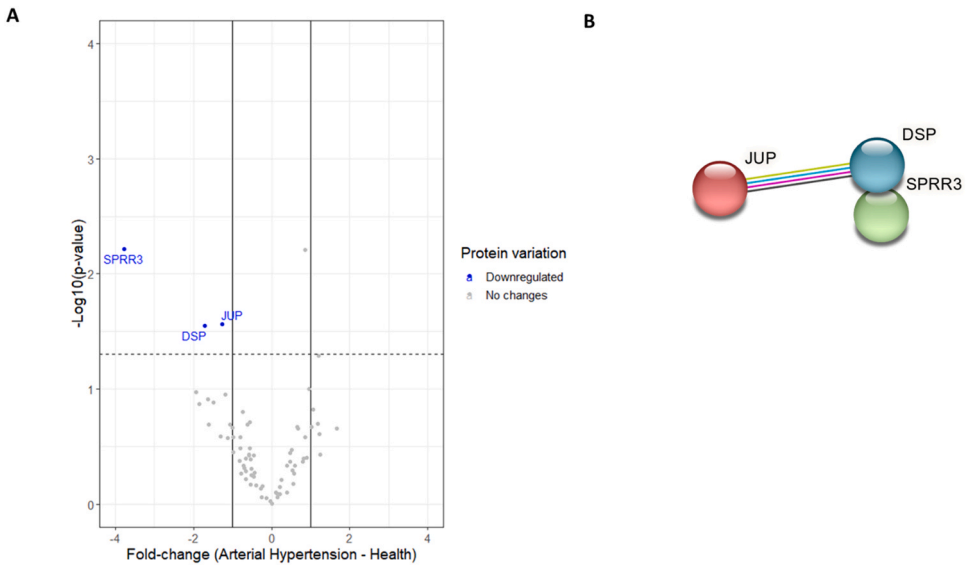

Fig. 1. LMW salivary proteome differences in the 3rd trimester of pregnancy per study group (A). proteins downregulated in AH women are highlighted in blue, namely small proline-rich protein 3 (SPRR3), desmoplakin (DSP), and junction plakoglobin (JUP). STRING network analysis of the downregulated proteins (B).

Table 2
Spearman correlations between the downregulated proteins in women with AH (small proline-rich protein 3 (SPRR3), desmoplakin (DSP), and junction plakoglobin (JUP)) and clinical oral parameters.

Gene names	DMFS	% Sites with CAL> 3 mm	BOP	PI
SPRR3	R= −0.16; p = 0.57	R= −0.15; p = 0.58	R= −46; p = 0.086	R= −0.35; p = 0.21
JUP	R= −0.42; p = 0.10	**R= −0.75; p = 0.001***	**R= −0.58; p = 0.024***	R= −0.18; p = 0.52
DSP	R= −0.35; p = 0.18	**R= −0.72; p = 0.002***	R= −0.50; p = 0.057	R= −0.17; p = 0.55

* $p < 0.05$

DSP-derived peptides showed significant moderate negative correlations with the oral health of the participants in the 3rd trimester of pregnancy, remarkably with the percentage of places with CAL > 3 mm (Table 3). The two DSP peptides significantly decreased in the saliva of AH participants also correlated negatively with BOP. Of note, the peptides from JUP and DSP that correlated negatively with parameters of periodontal health are localized in domains that interact with other proteins of the desmosome complex, such as plakophilin 1, plakophilin 2, desmocollin 1, and desmoglein 1 (Supplementary File 1).

3.4. Oral microbiota profiles

The samples used for proteome analysis were also characterized regarding their oral microbiota composition in the 3rd trimester and six months after delivery. After removing samples with less than 1000 reads, 39 samples remained in the dataset, which contained 1138

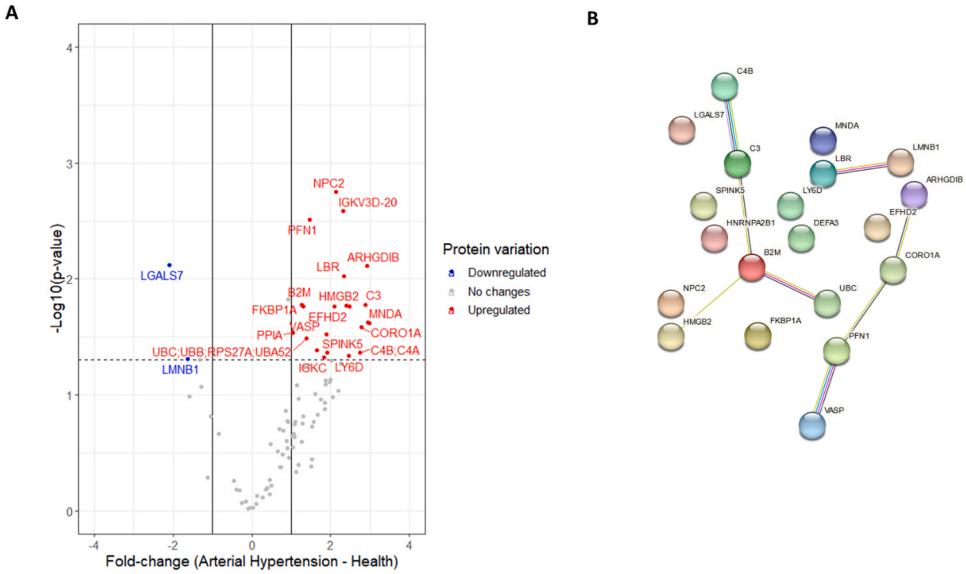

Fig. 2. LMW salivary proteome differences postpartum per study group (A). proteins downregulated in AH women compared to healthy women are highlighted in blue, whereas proteins upregulated are in red. String network analysis of the up and downregulated proteins (B). for the full name of the dysregulated proteins, see supplementary table 3.

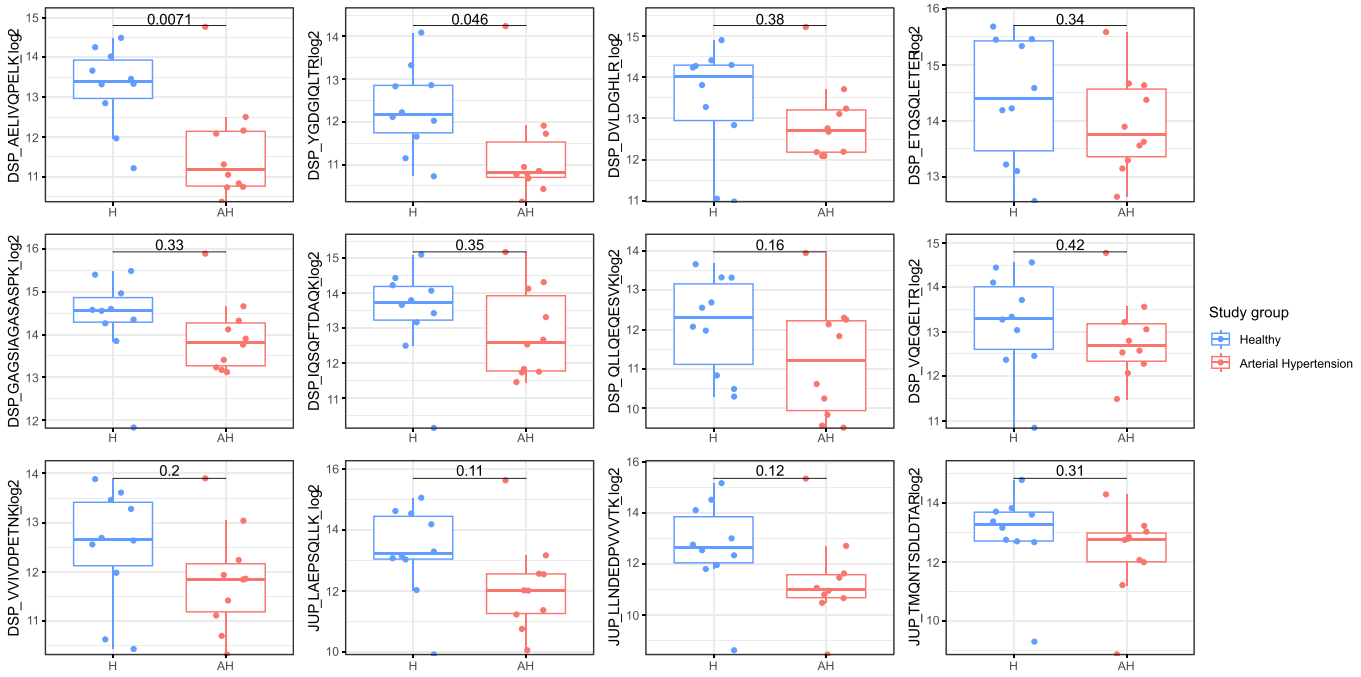

Fig. 3. Boxplot of the quantification of the peptides by parallel reaction monitoring (AH – Arterial hypertension; H – Healthy) (Mann-Whitney *u* test).

Table 3
Significant correlation of the peptides with oral health parameters of all women in the 3rd trimester of pregnancy.

Proteins	Peptides	Places with CAL> 3 mm	BOP
DSP	AELIVQPELK	R= −0.52; p = 0.045	R= −0.53; p = 0.042
	YGDGIQLTR	—	R= −0.53; p = 0.043
	DVLDGHLR	R= −0.61; p = 0.016	—
	ETQSQLETER	—	—
	GAGSIAGASASPK	R= −0.55; p = 0.034	—
	IQSQFTDAQK	R= −0.58; p = 0.023	—
	QLLQEQESVK	R= −0.59; p = 0.021	—
	VQEQELTR	—	—
	VVIVDPETNK	R= −0.52; p = 0.049	—
JUP	LAEPSQLLK	R= −0.61; p = 0.017	—
	LLNDEDPVVVTK	R= −0.57; p = 0.025	—
	TMQNTSDLDTAR	R= −0.60; p = 0.018	—

zOTUs, classified in 108 (known) genera, 55 families, and 10 phyla. We observed a significantly higher Shannon diversity in women with AH six months after delivery (Mann-Whitney *U* test; p = 0.013) (Fig. 4).

The oral microbiota composition in the 3rd trimester significantly differed between the groups in the 3rd trimester (PERMANOVA; F=1.63, p = 0.02) (Fig. 5 A). Likewise, there were differences in the oral microbiota composition six months after delivery (PERMANOVA; F=1.58, p = 0.03) (Fig. 5B). The oral microbiota of healthy and hypertensive women was dominated by *Streptococcus*, *Prevotella*, and *Veillonella* at both time points (Fig. 6). However, during the 3rd trimester of pregnancy, *Neisseria* (ALDEx2; effect=0.89, p = 0.015), *Fusobacterium* (effect=0.78, p = 0.025), *Rothia* (effect=0.74, p = 0.033), *Capnocytophaga* (effect=0.59; p = 0.033), and *Lautropia* (effect=0.75, p = 0.011) were lower in AH women. Six months after delivery, *Neisseria* (ALDEx2; effect=1.09, p = 0.0014), *Gemella* (effect=0.92, p = 0.017), *Bergeyella* (effect=1.019, p = 0.0045), *Streptococcus* (effect=1.00, p = 0.0062), *Rothia* (effect=0.92, p = 0.0017), *Haemophilus* (effect=0.71, p = 0.041), and *Capnocytophaga* (effect=0.84, p = 0.031) had a lower relative abundance in AH women. Conversely, *Dialister* (effect=-0.68, p = 0.017) had a higher relative abundance in AH women.

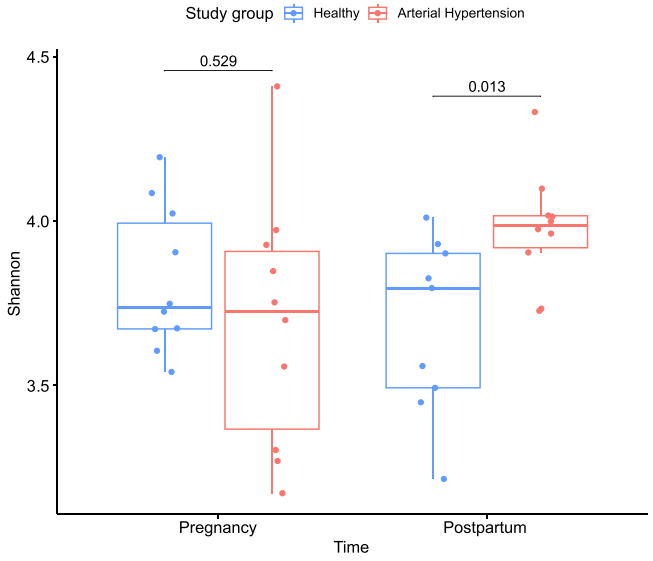

Fig. 4. Shannon diversity of both study groups in the 3rd trimester (pregnancy) and 6 months after delivery (postpartum) (Mann-Whitney *u* test).

3.5. *Correlation between the oral microbiota and peptide datasets*

To assess if there was a correlation between the salivary LMW proteome and microbiota, we evaluated the correlation between the validated peptides with oral genera that were differentially abundant in the 3rd trimester of pregnancy (ALDEx2; p < 0.05). We observed significant positive correlations between two (of three) JUP peptides and three (of nine) DSP peptides and *Neisseria*, between one DSP peptide and *Rothia*, one DSP and one JUP peptides and *Capnocytophaga*, and one JUP peptide and *Lautropia* (Table 4).

4. Discussion

Pregnant women with AH presented decreased levels of some

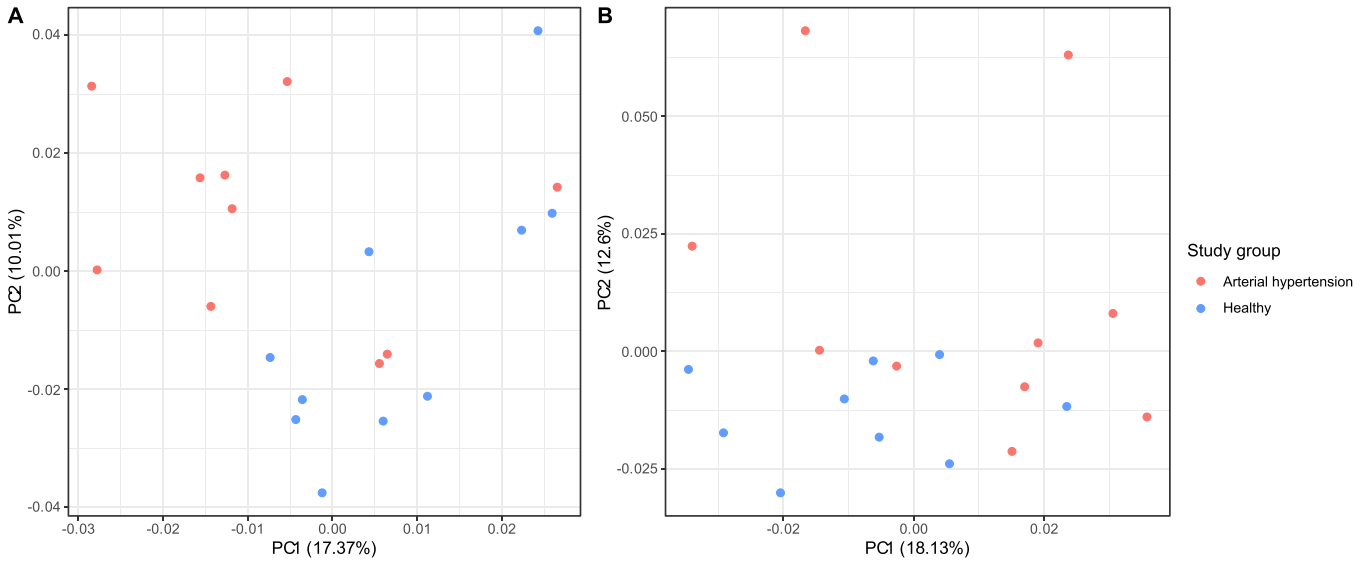

Fig. 5. Principal component analysis ordination of samples from the women in the 3rd trimester of pregnancy (A) and six months postpartum (B). differences in microbial composition were observed at both time points (PERMANOVA; 3rd trimester: F=1.79, p = 0.022; 6 months after delivery: F=1.90, p = 0.012).

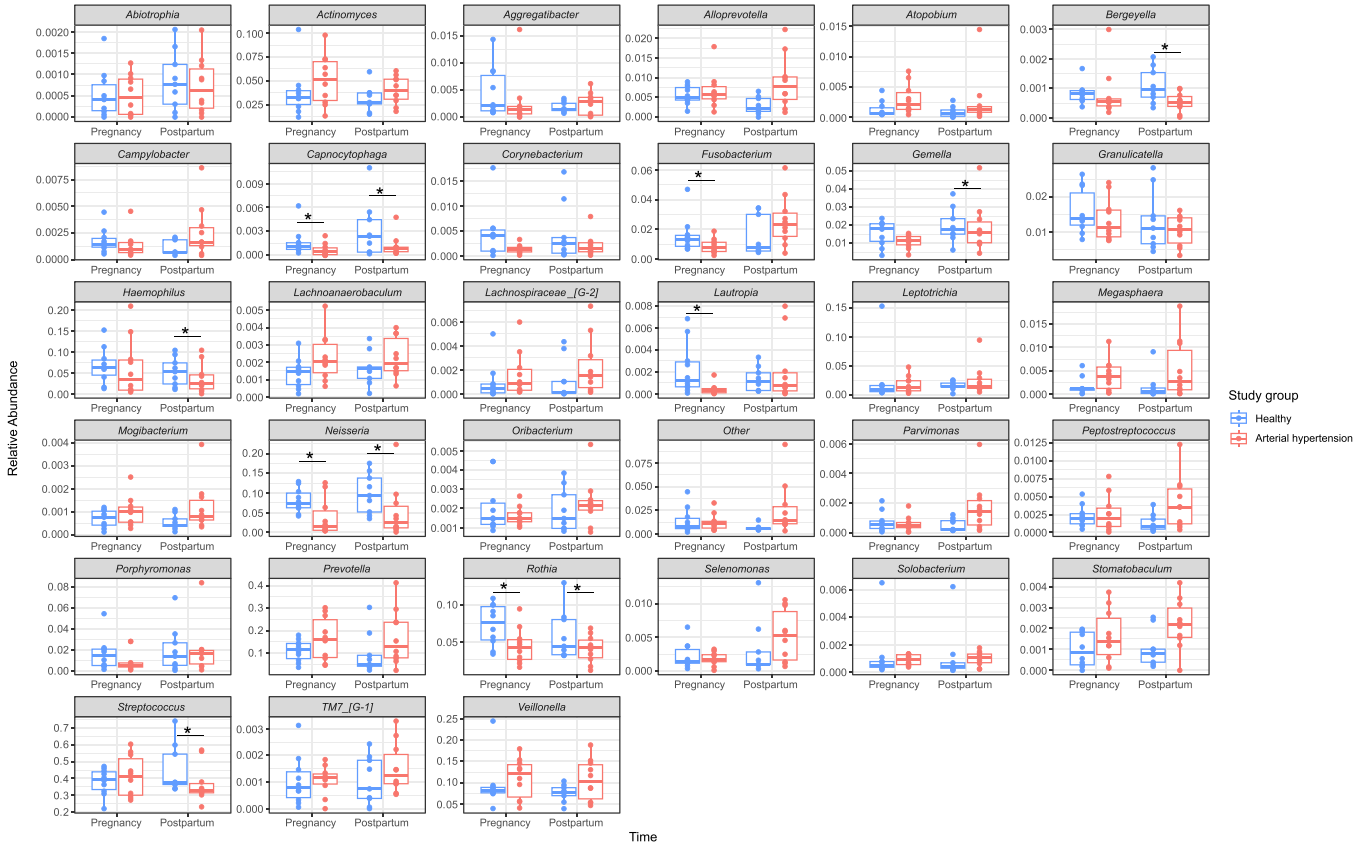

Fig. 6. Boxplots of the relative abundance of the genus-level composition of the oral microbiota by study group and time point. "Other" Contains the genera at a relative abundance of < 0.05 % with a prevalence of < 50 %. the asterisk (*) identifies the differentially abundant genera between groups, according to ALDEx2.

salivary proteins or their fragments, namely junction plakoglobin (JUP), desmoplakin (DSP), small proline rich protein 3 (SPRR3), and galectin 7 (LGALS7). Furthermore, pregnant women with AH scored significantly higher in the assessed oral health parameters, namely DMFS, BOP, CAL, and PI, displaying more gingival inflammation. Both JUP and DSP, quantified in the low molecular weight proteome, correlated negatively with oral health parameters, such as previous caries experience and periodontal health of the pregnant women. After validation of the peptides by parallel reaction monitoring, similar correlations were observed between JUP and DSP peptides and periodontal health parameters of the study population. Moreover, the oral microbiota diversity of women with AH was also significantly higher in the postpartum and the composition was different from healthy women at both timepoints. Some genera, namely *Fusobacterium*, *Neisseria*, *Rothia*,

Table 4
Results of the spearman correlation between microbial genera that had a significantly different relative abundance between healthy and AH pregnant women (ALDEx2) and the quantified peptides in the 3rd trimester of pregnancy.

Protein	Peptides	*Neisseria*	*Rothia*	*Capnocytophaga*	*Lautropia*
DSP	AELIVQPELK	R= 0.47; p = 0.037	R= 0.53; p = 0.016	—	—
	YGDGIQLTR	—	—	—	—
	DVLDGHLR	—	—	—	—
	ETQSQLETER	—	—	—	—
	GAGSIAGASASPK	—	—	—	—
	IQSQFTDAQK	R= 0.46; p = 0.043	—	—	—
	QLLQEQESVK	R= 0.49; p = 0.028	—	R= 0.47; p = 0.039	—
	VQEQELTR	—	—	—	—
	VVIVDPETNK	—	—	—	—
JUP	LAEPSQLLK	—	—	—	—
	LLNDEDPVVVTK	R= 0.46; p = 0.038	—	R= 0.45; p = 0.047	R= 0.47; p = 0.035
	TMQNTSDLDTAR	R= 0.49; p = 0.047	—	—	—

Capnocytophaga, and *Lautropia*, were less abundant in women with AH, and the two latter correlated positively with peptides from JUP and DSP.

Pregnant women with AH scored higher in the evaluated oral health parameters in comparison to normotensive women, similarly to what was observed by other studies (Czesnikiewicz-Guzik et al., 2019; Kawabata et al., 2016). Some authors hypothesize that endothelial function and systemic inflammation may link these two factors, but the mechanism is not yet understood (Czesnikiewicz-Guzik et al., 2019; Li et al., 2011; Tonetti et al., 2007). Moreover, we found that AH is associated with reduced levels of peptides from small proline rich protein 3 (SPRR3), desmoplakin (DSP), and junctional plakoglobin (JUP) in saliva LMW proteome. DSP is the most abundant protein of the desmosome structure (Yuan et al., 2021). It is co-expressed with JUP, a member of the catenin family of proteins. Together, these proteins are key for desmosome organization and epithelial cell-cell adhesion, and their downregulation is implicated in the loss of epithelium integrity (Fang et al., 2020; Huda Raza & Ghafoor, 2020). The level of DSP and JUP is influenced by systemic and local inflammation, which is concurrent with AH (Müller et al., 2021) and the higher periodontal scores observed in our population. Irrespective of the approach, we found that these two proteins and their respective peptides were reduced in women with AH. Although we could not confirm a reduction of these structural molecules at the whole protein level, previous studies have associated a decreased salivary level of DSP with periodontal disease (Belibasakis et al., 2015). These correlations reinforce the possible association between salivary proteome and oral health and how AH may influence both.

Six months after delivery, the profile of dysregulated proteins changed, and women with AH had an increase in pro-inflammatory proteins and a decrease in galectin 7 (LGALS7). In the oral cavity, increased LGALS7 is associated with increased wound healing and fibroblast proliferation (Belda-Ferre et al., 2015; Huang et al., 2022). Also, galectin 7 is anti-inflammatory and contributes to intracellular immunity against bacterial infection (Sewgobind et al., 2021). This could reflect immune system adaptations that happen during pregnancy and normalization after delivery (Abu-Raya et al., 2020). In this study, the oral examination was performed only once, during pregnancy. Although our findings suggest that women with AH may also have higher oral inflammation in the postpartum, this needs to be confirmed by clinical oral examinations.

The bacterial alpha diversity of the saliva from AH women was significantly higher than that of healthy women. This higher diversity in women with AH is concurrent with a higher plaque index observed in this study group during pregnancy. In fact, high bacterial diversity has been suggested to play a role in oral diseases because it may indicate a more complex biofilm (Nath et al., 2022; Ribeiro et al., 2022). Microbial composition was also different between healthy women and women with AH both in pregnancy and postpartum. This suggests that oral dysbiosis in AH-women may be independent of the pregnancy status. The lower relative abundance of health-associated genera, such as *Neisseria*, *Streptococcus,* and *Capnocytophaga,* further reinforces the idea of oral dysbiosis in women with AH (Hiranmayi et al., 2017). Of note, *Neisseria, Capnocytophaga,* and *Rothia* had a lower relative abundance in AH regardless of the pregnancy status, and this has been previously reported in AH patients (Barbadoro et al., 2021). These genera are known for reducing nitrates into nitrite, which contributes to oral nitric oxide (NO) production (Pignatelli et al., 2020; Rosier et al., 2022). Besides its blood pressure-modulating properties, NO can also promote tissue regeneration and has anti-inflammatory effects. This could justify the increased oral inflammation observed clinically and in the oral proteome of women with AH (de Farias et al., 2020; Pignatelli et al., 2020). Combined, this may explain why these genera are decreased in hypertensive women.

We observed positive correlations between DSP and JUP peptides, which can be regarded as markers of desmosomal integrity, and oral genus *Neisseria*. One hypothesis arising from this study is that AH-driven changes in the oral microenvironment may increase the susceptibility of women with AH to a loss of clinical attachment and, thus, a deterioration of the desmosomal integrity, higher bleeding on probing, and oral inflammation, and ecological shifts in the oral microbiota. As such, the oral cavity of women with AH presents a higher number of anaerobic sites (periodontal pockets), and microorganisms such as *Neisseria* (strictly aerobe) and *Rothia* (facultative anaerobe) may be consequently reduced. These ecological shifts might further contribute to the dysregulation of blood pressure in AH. We cannot dissect the causal effect in our study, but our findings suggest a possible interplay between arterial hypertension, oral dysbiosis, and the salivary proteome.

Some important limitations must be addressed regarding our study. First, the low number of samples may mask significant changes in the oral LMW proteome or microbiota. For this reason, it would be important to validate these findings in a larger cohort in the future. The differences found did not overlap between the two approaches (whole proteome and LMW proteome). For instance, both JUP and DSP are high molecular weight proteins but were only reliably detected in the LMW proteome. One of the reasons might be the unspecific proteolysis of proteins in saliva. For this reason, the prediction of protease activity in saliva samples (without the masking effect of trypsin digestion) might provide further insights related to native proteome/peptidome (Trindade et al., 2018). Moreover, the follow-up of our cohort was impaired by the COVID-19 pandemic, namely regarding the possibility of performing oral health assessments in postpartum and in some healthy women during pregnancy. It would have been important to do this to confirm if women with AH have lower oral health status, regardless of the pregnancy status. Finally, with our PRM approach, we quantified peptides and, in future studies, it would be important to validate if the corresponding whole proteins are also altered in the whole salivary proteome.

Despite these limitations, our results open new paths for further research on the interplay between oral and systemic health by correlating data from two "OMICS" with clinical parameters. In the future, the relationship between the proteins identified in this study and arterial

hypertension should be further explored in a population of hypertensive participants with a healthy periodontium. Moreover, this study may pave the way to *in vitro* experiments on the mechanisms behind the observed correlations of specific oral genera, such as *Neisseria*, with desmosome proteins in the oral cavity and their contribution to oral dysbiosis and systemic health.

In summary, AH seems to play an essential role in the oral health of pregnant women and may modulate their oral proteome and microbiota. The findings of this exploratory study justify further research to confirm and further clarify the mechanisms underpinning the relationship between AH and oral health.

Ethics approval and consent to participate

The project was approved by the ethics committee of *Centro Hospitalar Universitário de São João*, Portugal (CHUSJ) in December 2018 (Nº294/2018) and by *Unidade Local de Saúde de Matosinhos*, Portugal (ULSM) (86/CE/JAS), with authorization for the reuse of Hospital Clinical Records for Research. Written informed consent was obtained from all participants.

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CRediT authorship contribution statement

Benedita Sampaio-Maia: Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Fábio Trindade:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ana Filipa Ferreira:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Maria João Azevedo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Egija Zaura:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Bernd W. Brandt:** Writing – review & editing, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mark J. Buijs:** Writing – review & editing, Validation, Methodology, Investigation. **Petr Novák:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Dmitry Loginov:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Data curation. **Carla Ramalho:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Inês Falcão-Pires:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.archoralbio.2025.106319.

Data availability

Data used for analyses in this study are available upon request.

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