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Altered salivary miRNA and microbial profiles reflect different responses to psychosocial stress

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Abstract

Psychosocial stress is a major risk factor for mental and physical illness, with emerging evidence pointing to oral microRNAs (miRNAs) and the microbiome as potential biomarkers.

This study investigated stress-associated molecular changes in saliva from 113 male police officers stratified by perceived stress response (SR) into low, intermediate, or high responders. Salivary miRNA profiles were analyzed using small RNA sequencing, and microbiome composition was assessed through shotgun metagenomics.

Eighteen miRNAs were dysregulated between high- and low-SR groups and reported a progressive alteration from low- to high-SR groups. Functional enrichment analysis indicated that dysregulated miRNA targets were involved in apoptosis, cellular stress responses, and metabolic regulation.

Distinct alterations in salivary microbial communities were observed alongside SR levels. Functional analysis indicated enhanced inositol degradation and reduced pathways for L-tryptophan and thiamine biosynthesis in high-SR individuals.

These findings suggest that salivary miRNAs and microbiota may serve as putative non-invasive biomarkers of psychosocial stress and provide insight into mechanisms linking chronic stress to physiological and behavioral outcomes.

Introduction

Stress is an engineering term that refers to the tension or load to which a material is subjected. In medicine and biology, it represents the stimulus that elicits the general homeostatic response in living organisms[1,2]. This response to real or perceived environmental, psychological, or physiological threats triggers coordinated neuro-immuno-endocrine processes to maintain or restore homeostasis[2]. Depending on stress intensity, duration, predictability, and individual coping capacity, the stress response (SR) may elicit distinct physiological and behavioral reactions. These responses could be adaptive, leading to habituation (eustress), or ineffective and even maladaptive, such that the organism fails to return to physiological and/or psychological homeostasis (distress) and may be exposed to altered mental and physical health[3]. It is well known that there can be stress without distress[4]: in other words, exposure to the same stressor can have divergent effects in different individuals, being positive for one, neutral for another, and negative for a third. Modern theories of stress focus on people's interactions with their work environment, on the psychological mechanisms underlying these relationships, or on the transactional mechanisms of cognitive appraisal and coping. Distress is not yet a disease, but it can become one. Indeed, when SR demands exceed available resources, there could be an increase in the risk for the development of acute or chronic diseases, including infection, cardiovascular, metabolic, and mental diseases, as well as cancer[5].

Advances in understanding the basic regulatory mechanisms of SR have revealed the role of microRNAs (miRNAs), small non-coding RNAs approximately 22 nucleotides long[6,7]. miRNAs modulate the expression of protein-coding genes at the post-transcriptional level by binding to their target messenger RNAs and inducing their degradation or translational repression. miRNAs can be detected in extracellular fluids, including serum, plasma, and saliva. Accumulating evidence indicates aberrant miRNA expression in several human diseases[6]; therefore, miRNAs may serve as potential biomarkers and signaling molecules in pathogenic pathways. The role of miRNAs in stress signaling was recognized early in loss-of-function studies, in which experimental animals with miRNA knockout or inactivation normally developed but were unable to cope with stress[8]. Human studies have confirmed the involvement of miRNAs in the regulation of acute and chronic SR[6,7,9–12], underscoring their potential as biomarkers for the diagnosis, prevention, and monitoring of stressful conditions.

At the same time, thanks to deep sequencing and sophisticated computational analyses, the ability to comprehensively investigate the human microbiome has, over the last decade, led to numerous studies on the relationship between microbial species and diseases[13]. Research has also revealed many associations between gastrointestinal tract microbes and stress/SR (anxiety and depression in pregnant or postpartum women, or posttraumatic stress disorders)[14]. The hypothesis of the gut-brain axis has been corroborated by direct interactions between intestinal microbes and changes in mental health status, specific disorders, or general stress. More recently, the study of the oral microbial composition has emerged as important for investigating the relationships among multifaceted aspects of stress, the host, and the microbes that interact with it[15,16].

Work-related distress is a chronic psychosocial condition that occurs “when the demands of the work environment exceed the employees’ ability to cope with (or control) them”[17], or when there is a discrepancy between the efforts made to work and the rewards received for the work done[18]. Distress is the second most common work-related health issue in Europe, leading to anxiety, depression, fatigue, worse quality of life, physical illness, as well as reduced participation, performance, and safety at the workplace[19].

Peculiar groups of workers, such as public safety personnel and first responders, including police, firefighters, and emergency dispatchers, are at high risk of potential work-related mental health issues as a consequence of psychosocial distress[20]. Police officers may be significantly exposed to both operational work-related challenges (e.g., violence and trauma, work shift, working hours) as well as to organizational stressors (e.g., negative public perception of police, perceived lack of departmental support, interface with the judicial system, bureaucracy)[21]. Greater perceived work stress was associated with a higher risk of developing or aggravating mental and physical health problems, including sleep problems, anxiety, depression, musculoskeletal disorders, metabolic syndrome, cardiovascular disease and cancer[22–25]. An umbrella review, covering data on police officers from 43 countries, identified twenty-six adverse health outcomes and 220 underlying risk factors, 136 of which

were modifiable, including work-related stress[26]. Moreover, work-related stress also reduced well-being[27] and work performance, thus threatening public safety[28].

The present study aimed to evaluate the association between chronic psychosocial SR, salivary miRNA profiles, and the oral microbiome. As a model of psychosocial stress, we leveraged the longitudinal Genoa Police Cohort, a census of workers in service in the mobile department of Genoa (Italy) in 2009, which was monitored for SR over thirteen years until 2022[29]. Our hypothesis was that individuals grouped by SR (low, intermediate, and high distress) also differ in their salivary miRNome and microbiome, reflecting their different levels of resilience.

Results

Subject characteristics

Police officers were divided into three groups based on SR scores (low-, intermediate-, and high-SR), and their relative sociodemographic and occupational characteristics are shown in **Table 1**. No significant differences in the distribution of these characteristics were observed among the three study groups stratified by SR levels, except for age. Participants in the high-SR group were in fact younger than those in the low- and intermediate-stress groups (p=0.04). This difference may be attributable to the early withdrawal from the cohort by subjects with high SR. Age was then considered as a covariate in the subsequent analyses.

Salivary miRNAs are differentially expressed in different stress response groups

miRNome profiled in saliva samples by small RNA-seq resulted in an average of 44,243 reads aligned on human miRNA annotations and an average of 254 miRNAs detected in each sample (range:186-549) (**Supplementary Table 1A**). The most abundant salivary miRNA identified was miR-148a-3p, followed by miR-526a-5p, miR-520c-5p, miR-518d-5p, miR-3135a-3p, miR-223-3p, and miR-1246. An age-adjusted differential expression analysis was performed by comparing high-versus low-SR, as well as intermediate-versus low-and intermediate-versus high-SR groups. In the comparison high- versus low-SR, 18 miRNAs displayed significant different levels among groups, with four (miR-10400-5p, miR-1290, miR-6074-5p, and miR-9902) and fourteen (miR-203a-3p, miR-7-5p, miR-143-3p, miR-181a-5p, miR-142-5p, miR-223-5p, let-7f-5p, let-7g-5p, miR-26b-5p, miR-27a-3p, miR-30e-5p, miR-26a-5p, miR-142-3p, and miR-21-5p) miRNAs characterized by higher and lower levels, respectively, in the high-SR group (**Figure 1A** and **Supplementary Table 1B**).

On the other hand, only miR-143-3p levels significantly decreased in the intermediate group when compared with the low-SR group, while nine miRNAs (miR-10400-5p, miR-558-5p, miR-27b-3p, miR-221-3p, miR-148a-3p, miR-22-3p, miR-203a-3p, miR-27a-3p, miR-21-5p) were associated with significantly different levels between the intermediate and the high-SR group. In this last set, the levels of miR-10400-5p and miR-558-5p were increased in the high-SR group, and those of miR-27b-3p, miR-221-3p, miR-148a-3p, miR-22-3p, miR-203a-3p, miR-27a-3p, and miR-21-5p decreased (**Figure 1A-B**). Interestingly, the

levels of four miRNAs (miR-10400-5p, miR-203a-3p, miR-27a-3p, and miR-21-5p) were commonly altered across multiple comparisons and showed a progressive increase or decrease from the low- to the high-SR group (**Figure 1C**).

miRNA target enrichment analysis

Functional analysis of the identified DE miRNA target genes (**Figure 2A** and **Supplementary Table 1C-D**) resulted in an enrichment in genes involved in apoptosis-related processes, cellular response to stress, negative regulation of gene expression, responses to abiotic stimuli, positive regulation of catabolic processes, and macromolecule catabolic processes. Network analysis of the miRNA-target interactions supported by the highest number of evidence showed miR-21-5p as the main miRNA hub, followed by miR-223-5p, and miR-27b-3p/miR-27a-3p. Several genes were targeted by multiple miRNAs with decreasing levels in the high-SR group, including *PTEN*, *PITHD1*, *MDM2*, *MAT2A*, *KPNA2*, *FIGN*, *CDKN1B*, *CCND1*, and *BCL2*, each targeted by three different miRNAs (**Figure 2B** and **Supplementary Table 1E**).

Salivary metagenome profiles

Shotgun metagenomic sequencing was performed on the same set of samples analyzed for miRNAs. After the removal of host DNA content, an average of 18.3±8.4 million reads were obtained, of which an average of 26.53% were assigned to microbial annotations, resulting in 237.0±82.9 SGBs detected across samples (**Supplementary Table 2A**). Microbial diversity analysis among the three study groups did not show any significant difference in terms of alpha diversity (Richness, Shannon index, Inverse Simpson index and Evenness) (**Supplementary Figure 1A**), as well as on the beta diversity computed among samples (PERMANOVA $p>0.05$) (**Supplementary Figure 1B**).

The differential abundance analysis showed two microbial classes, three orders, two families, 13 genera, 44 species, and 46 SGBs with a significantly different abundance among the study groups ($p<0.05$), with the comparison between high-SR and low-SR associated with the highest number of differential taxa (n=62) (**Supplementary Figure 1C** and **Supplementary Table 2B**). Considering the SGB-level results, 15 taxa increased and 30 decreased from the low- to the high-SR group (**Supplementary Figure 1C**). Focusing on taxa detected in >50% of samples, *Prevotella baroniae*|SGB1533and *Schaalia odontolytica*|SGB17169 were characterized by the most significantly increased levels in the high-SR group, while *Actinomyces naeslundii*|SGB15888, and *Capnocytophaga ochracea*|SGB2497were decreased (**Figure 3A** and **Supplementary Table 2B**).

The analysis of the microbial pathway abundance estimated by the sequencing data also showed six pathways differentially enriched among the study groups (**Figure 3B** and **Supplementary Table 2C**). Among them, the *Myo-, chiro- and scyllo-inositol degradation* (PWY-7237) was characterized by the most significant increase in the high-SR group, while *L-tryptophan biosynthesis* (TRPSYN-PWY) was characterized by the most significant decrease. Conversely, no pathways were significantly altered between the high- and intermediate-SR groups (**Figure 3B** and **Supplementary Table 2C**). A deep analysis of the microbial species contributing to the differentially abundant pathways showed that the increase of

genes involved in the *Myo-, chiro- and scyllo-inositol degradation* (PWY-7237) was mainly driven by the increase of Actinomyces, Haemophilus, Oribacterium, and Rothia bacteria (**Figure 3C** and **Supplementary Table 2D**). On the other hand, in the high-SR group, the decrease in genes involved in *L-tryptophan biosynthesis* was primarily driven by reductions in Aggregatibacter, Neisseria, Streptococcus, and Veillonella.

Integrative analysis of miRNA and metagenomic profiles

To identify the subset of molecular features (salivary miRNAs, SGBs, and microbial pathways) with the highest discriminatory potential for the study groups, an integrative analysis using DIABLO was performed. The analysis showed that the microbial pathways were associated with the greatest potential for separating the high- from the low-SR groups (**Figure 4A**), whereas the variate computed from miRNA and SGB levels was correlated (**Figure 4B**). Among the most informative features from each molecular layer, miR-142-3p, miR-7-5p, and miR-143-3p resulted in the three most informative miRNAs, while *GGB12783 SGB19821*|SGB19821, *Prevotella enoeca*|SGB1527, and *GGB2676 SGB3611*|SGB3611 were the top bacteria (**Figure 4C**). Among the microbial pathways, *Myo-, chiro-, and scyllo-inositol degradation* (PWY-7237), *UTP and CTP dephosphorylation I*, and the *Thiazole component of thiamine diphosphate biosynthesis* (PWY-6892) were the most informative. The impact of these microbial pathways on separating the study groups was clearly highlighted by the multi-omic clustering analysis, which showed that the high- and intermediate-SR groups were distinct from the low-SR group (**Figure 4D**).

Discussion

In the present study, salivary miRNA and microbial profiles were investigated in a cohort of otherwise healthy police officers, well characterized for their occupational exposure and stress responses. Despite the small sample size, the data confirmed that psychosocial stress is associated with changes in salivary miRNA profiles and the oral microbiome. For the first time, it was shown that high levels of psychosocial SR are characterized by a specific salivary miRNA expression pattern as well as changes in candidate microbial pathways that showed a sort of dose-dependent increase/decrease compared with those of resilient subjects with low-SR.

We chose saliva as a sampling matrix because it is not only non-invasive and easily collected and managed, but it has also proven useful for assessing miRNAs and microbial species as biological markers across different pathophysiological conditions, including stress[16,30].

miRNAs have emerged as an integral part of SR for many years[6,8,10,31,32], and their role as biomarkers of both acute[9,11,12,30] and chronic[9,11] psychological stress has been reported, either in experimental or naturalistic settings. Although there is growing interest in the relationship between miRNAs and stress, occupational studies in this field are few. Literature demonstrated that work-related stress is consistently associated with an increased risk of mental and physical issues[33]. To the best of our knowledge, there

are no investigations on miRNA profiling conducted on longitudinal occupational cohorts, in which potential confounding variables were mostly known and controlled, and SRs were monitored for several years, demonstrating longitudinal stability of the individual differences in physiological response to work-related stressors.

The observed alterations in miRNAs between high- and low-SR groups either confirmed known stress-associated miRNA changes or identified a new miRNA set associated with distress. Notably, miR-21-5p expression has already been linked to human psychological SR, and accordingly with our results, salivary miR-21-5p levels were decreasing under acute stress test (the Trier social stress test)[30] and in whole blood under chronic stressful event (academic exam)[34]. miR-21-5p is among the most studied miRNAs, is expressed in most cell types, and is involved in several stress-related processes, including apoptosis and inflammation. It is frequently overexpressed in neoplastic, inflammatory, cardiovascular, and neurodegenerative diseases, as well as type 2 diabetes[35]. Although the mechanisms leading to miR-21-5p decrease in highly distressed subjects are presently unknown, we can speculate a role for sustained sympathetic activation with miR-21-5p downregulation. Indeed, the stress mediator adrenaline, released upon sympathetic nervous system activation, reduces miR-21 expression in human bone marrow-derived stem cells[36]. A significant correlation has been found between salivary alpha-amylase, a surrogate marker for sympathetic activation, and salivary miR-21 levels under acute psychological stress[30]. miR-21-5p has also emerged as an inflammamiR, since it is commonly up-regulated by inflammatory and pro-oxidant stimuli, and exerts compensatory anti-inflammatory and anti-apoptotic actions by targeting components (*PTEN* and *PDCD4*) of inflammatory pathways such as the nuclear factor kappa B (NF-κB) pathway[37]. Notably, miR-21-5p also targets *A20*, an inhibitor of NF-κB activation, suggesting a possible context- and time-dependent regulation of inflammation[38]. Beyond its specific cellular or physiological functions, miR-21-5p downregulation in highly stressed individuals may indicate an uncompensated and overactive SR, potentially predisposing to adverse health outcomes.

Salivary miR-26a-5p and miR-26b-5p levels were also decreased in high-SR police officers. They have been previously associated with psychological stress[9,30]. miR-26a-5p is highly expressed in the central nervous system, where it regulates synaptic plasticity, neuronal morphogenesis, neurotransmission, and axon regeneration[39]. Some studies have reported increased expression of miR-26a-5p and miR-26b-5p in whole blood and of miR-26b-5p in saliva under stress conditions[9,30]. However, there may be obvious differences in types, duration, and intensity of stressors between different studies, and in the case of the present investigation, any observed changes in miRNA levels and patterns were associated with (and potentially driven by) long-standing high stress, which may imply unique molecular adaptations reflecting established resilience or vulnerability phenotypes.

A decrease of salivary let-7g-5p and let-7f-5p levels was also observed in the high-SR group. These miRNAs are members of the large let-7 family and have previously been reported to be downregulated in whole blood in neurodegenerative diseases[40]. In contrast, let-7g-5p levels increased in subjects with chronic fatigue syndrome, sleep disorders, mood swings, headaches/migraines, depression, anxiety, and

other likely stress-related diseases[11]. Among other differentially expressed miRNAs, miR-223-5p has been reported to exert a neuroprotective effect[41]. Its downregulation in the high-SR group (as observed by us) may therefore contribute to chronic stress-induced excitotoxicity and neuropathology[42].

In accordance with the results in saliva observed by us, the experimental acute stress (inescapable tail shock) in rats caused a sympathetic nervous system activation-mediated downregulation of plasma exosome miR-203a-5p and miR-142-5p[43], which are have effects on immune cell survival[44] and inflammation[45] miR-142-3p mediates neuroinflammation-induced synaptic dysfunction in mice[46] and is downregulated in immune cells of aged mice with associated elevation of proinflammatory factors, potentially contributing to "inflamm-ageing"[47]. Notably, the expression levels of miR-142-3p target gene *AFF1* were found to be upregulated in microglia (immune cells of the brain) of stressed mice compared with controls, as part of a transcriptional program regulating gene transcription, catabolism, and macromolecule biosynthesis, in line with increased metabolism and energy demand under chronic stress[48].

A previous study reported that miR-27a-3p and miR-30e-3p levels were decreasing in the blood of rats displaying vulnerability (increased anxiety-like behavior) to chronic stress (social defeat) compared with resilient rats[49]. Members of the miR-30 family, including miR30e-5p, have been proposed as tumor suppressors due to their ability to induce cellular senescence in response to DNA damage or other cellular stress[50].

Other miRNAs with decreasing salivary levels in the high SR group in this study were miR-181a-5p, miR-7-5p, and miR-143-3p; the first two have been previously reported to be involved in various types of cellular stress, including endoplasmic reticulum stress[37] and oxidative stress[51]. Conversely, miR-143-3p has been involved in the occurrence of depressive-like behaviors induced by chronic stress (chronic restraint stress) in mice, mediating alterations in synapse density in the hippocampus[52]. However, miR-143-3p might also exert anti-inflammatory and anti-apoptotic effects[53].

Some miRNAs were newly identified as responsive to high work-related stress in our study, including miR-9902, miR-6074-5p, miR-1290, and miR-10400-5p. Studies reported tumor-promoting effects[54] and a role in neuronal differentiation[55] for miR-1290.

In the comparison of high stress with intermediate stress, a significant specific deregulation was found in five miRNAs: miR-558-5p (with increasing levels), miR-27b-3p, miR-221-3p, miR-148a-3p, and miR-22-3p (all with decreasing levels). Moreover, a few miRNA sets were common to those altered under high versus low stress and followed the same pattern: miR-203-3p, miR-27a-3p, miR-21-5p (decreasing), and miR-10400-5p (increasing). This pattern might suggest early perturbation of salivary miRNAs in association with an intermediate level of work-related SR. The functions these miRNAs might play out in SR are presently unknown. Anti-inflammatory roles for miR-558-5p and miR-148a-3p were described[56].

Serum miR-221-3p and miR-27a-3p/miR-27b-3p were upregulated in subjects with posttraumatic stress disorder[57].

In contrast to the evidence on the relation between psychosocial stress and gut microbiota[14], a few preclinical and clinical studies have found that psychological stress can induce changes in the oral microbiome[16,58]. Our metagenomic analysis showed no significant differences in ecological metrics of salivary microbiota composition among subjects, with only a few SGBs exhibiting significant differential abundances across the investigated stress categories. Specifically, *Prevotella baroniae|SGB1533* and *Schaalia odontolytica|SGB17169* were more abundant in the high-SR group compared to the low-SR group. Both species have been previously reported to play a role in stress-related disorders, including anxiety and depression[59], with consistent altered oral levels of the Prevotella and Schaalia genus in subjects with psychological stress[60]. On the other hand, in our study, a decrease in oral-specific SGBs, including *Actinomyces naeslundii|SGB15888*, *Corynebacterium durum|SGB17008* and *Actinomyces sp oral taxon 448|SGB15872* was observed in the high-SR. This is consistent with a study in a murine model by Venkatraman et al.[61] showed a decrease in oral Actinobacteria in animals under prolonged psychosocial stress, associated with dysregulation of inflammation-related genes. In addition, *Veillonella rogosae* SGB6956 was another taxon associated with a consistent decrease in the high SR observed in this study. The Veillonella genus is highly abundant in the oral cavity[62]. These bacteria are among the initial colonizers of enamel and are pivotal in the formation and maintenance of the supragingival and subgingival biofilms[63]. A decrease in this genus in the oral cavity has been observed in association with various oral and periodontal diseases; however, the available data remain contradictory and require further investigation[63]. Corynebacterium was previously reported to be significantly reduced in the oral cavities of rats subjected to chronic restraint stress compared with controls[64].

Notably, analysis of microbial pathway showed a clear SR group separation based on putative metabolic processes: the *Myo-, chiro- and scyllo-inositol degradation* (PWY-7237) pathway had an increased activity in high-SR groups, while the *L-tryptophan biosynthesis* (TRPSYN-PWY), *Superpathway of thiamine diphosphate biosynthesis* (THISYN-PWY), and *Thiazole component of thiamine diphosphate biosynthesis* (PWY-6892) were decreasing with the SR severity.

Inositol is a type of cyclic sugar alcohol that acts as a precursor of cellular secondary messengers and plasmatic membrane components, whose metabolism is strictly related to microbial fermentative activity[65]. Myo-inositol is the most abundant inositol in mammalian cells and plays a pivotal role in regulating neuronal connections, particularly during development[66]. Animal studies have shown increased inositol brain levels in chronic psychosocial stress[67]. However, the microbial degradation pathway of inositol is primarily characterized in the context of the gut microbiota and its role in growth, energy conservation, and lipid synthesis, with both favorable and adverse metabolic outcomes associated with increased microbial inositol conversion[68]. No previous studies have correlated the inositol degradation pathway with oral microbiome and psychosocial stress.

In parallel, in the high-SR group, a decrease of microbial genes involved in the biosynthesis of the essential amino acid L-tryptophan was observed. The microbiota can influence L-tryptophan metabolism via several pathways[69], with consequences for both the host and the bacteria physiology. L-tryptophan is the precursor for the biosynthesis of the gastrointestinal and neuroendocrine transmitter serotonin (or 5-hydroxytryptamine). Peripheral serotonin is predominantly synthesized by colonic enterochromaffin cells and exerts multiple physiological functions in the periphery, including the regulation of gut motility and fluid secretion, energy metabolism, cardiac contraction, vascular tone, coagulation, and the immune response[70]. Moreover, gut-derived serotonin communicates bidirectionally with the brain's serotonergic system via the gut–brain axis[71]. Certain bacterial species, including *Corynebacterium*[72], possess the genomic potential to synthesize serotonin, whereas others can regulate host serotonin levels through microbiota-derived metabolites or cell components that affect serotonin biosynthesis in enterochromaffin cells. Accordingly, a reduction in *Corynebacterium* was observed in both the human saliva microbiome and the rat oral microbiome of individuals under high stress[64].

L-tryptophan can be metabolized by the gut microbiota into the neurotransmitter tryptamine, as well as indole and its derivatives, which are ligands of the aryl hydrocarbon receptor (AhR) and participate in the immune and intestinal homeostasis[69]. Recent findings have disclosed a neuroprotective role for a microbiome-derived indole against chronic psychosocial stress in a mouse model [73]. Tryptophan can also be degraded by host immune and intestinal cells and by some bacterial species through the kynurenine pathway, thereby generating kynurenine and downstream products, such as kynurenic acid, quinolinic acid, which play roles in inflammation, immune responses, and neurobiological functions[69]. Several reports have shown that chronic stress and depression trigger the kynurenine pathway through the induction of inflammation, thus diverting tryptophan metabolism from the production of serotonin or indoles to the synthesis of neuroactive/neurotoxic metabolites[74,75]. Although chronic stress is associated with dysregulated L-tryptophan metabolism in both the central nervous system and the periphery, with a contributory role for an altered gut microbiome[76], the implications of changes in oral microbiota composition and metabolism remain unclear[77].

Thiamine diphosphate is the active coenzyme form of thiamine (vitamin B1), which is crucial for energy metabolism and, consequently, for various host and bacterial metabolic and immunoregulatory processes, as well as for neurotransmitter synthesis and protection against oxidative stress. Certain bacterial strains have long been known to contribute to vitamin B1 synthesis and availability for both the host and bacteria[78]. The observed reduction of the metabolic pathway related to thiamine diphosphate biosynthesis in the oral microbiome of the present study could be interesting in the context of chronic stress, since studies have suggested a (neuro)protective role for thiamine against stress, anxiety, depression and mental health in general[79]. Moreover, decreased thiamine metabolic pathway as observed here can negatively impact the microbiome itself in a vicious cycle[80], thus potentially contributing to reshape the microbiota as observed in response to stress.

Our findings are unique because they focus on the oral microbiome, a less-explored niche compared with the gut microbiota. Therefore, although our study revealed specific changes in oral microbial composition and function, additional investigations are needed to elucidate the potential role of the oral-brain axis and the intrinsic neural, immune, and microbial pathways in the regulation of SR.

We cannot determine whether the changes in salivary miRNAs and microbial species observed here represent a compensatory protective response to stress or reflect maladaptive physiological and psychological responses. It is possible that both conditions play out in work-related SR. For instance, whether the differential expression of miRNAs in saliva results from regulated compensatory responses of tissues to stress or from uncontrolled cell release is currently unknown. We cannot make any speculation about the microbiome results, as most prior work focused on the gut microbiome rather than the oral microbiome, and there are fundamental differences in chemical and microbiological composition between the oral cavity and the gut (oral-gut barrier).

This study was limited by a small sample size. Although the size of the present study was comparable to that of other studies on miRNAs and microbiota profiling and SR, this limitation was balanced by the advantage of having studied a population exposed to intense levels of occupational stress, with repeated control of the response to stress and possible confounding factors (diet, physical activity). Monitoring the SR - and the associated psychological and clinical outcomes - for many years has allowed the measurement of the response to chronic occupational stress, data that has so far been absent in the literature. Another limitation of this study was the lack of female police officers in the cohort. We cannot, therefore, determine the influence of the hormonal environment on miRNAs and microbial species, nor can we study their relationships with stress hormones or inflammatory factors. Finally, we have limited knowledge of the mechanisms underlying deregulation and outcomes of stress-associated miRNAs/taxa. Larger prospective studies that include female workers would allow researchers to assess the clinical value of miRNA/microbial changes induced by psychosocial stress.

On the other hand, the strengths of the study derived from 1) the use of a high-throughput approach (small RNA-seq and metagenomics) to explore salivary miRNA and microbial profiles, which is not based on a priori selection of candidate features and allows the identification of novel miRNAs/taxa associated with response to stress; 2) the use of an homogeneous study sample, with all recruited policemen engaged in maintaining law and order, and exhibiting, at recruitment, levels of stress comparable to the baseline ones (several years before), thus representing a naturalistic setting for studying true chronic stress resilience or vulnerability.

The finding that an unfavorable response to work-related psychosocial stress may be associated with specific miRNA and microbial signatures is novel and offers a new perspective on the pathogenic mechanisms of stress and their associated risks and outcomes.

Conclusions

Our exploratory study in police officers reveals dysregulated miRNAs and differences in microbial abundancies associated with stress. These alterations may reflect epigenetic changes and dysbiosis associated with work-related stress and may be accompanied by related physiological and psychological perturbations. The study also provides evidence that miRNAs from human saliva and the oral microbiome could be used as an amenable, non-invasive, and objective monitoring tool, in addition to neuropsychiatric evaluation, for early diagnosis, prevention, and evaluation of therapy for stress and future stress-associated diseases.

Methods

Study cohort

The present study was a cross-sectional analysis conducted in 2022 on the Genoa Police Cohort composed of police workers from the “VI Reparto Mobile” (Mobile Unit) of Genoa (Italy), who were in service in 2009 (n = 292) and remained in the same unit until 2022 (n = 113). Members of the mobile departments are specialized and responsible for maintaining public order and performing work widely recognized as physically and psychologically demanding. They carry out the same public-order tasks regardless of their qualifications, rank, or seniority. Workers must maintain strict control of their lifestyle, and their preparation includes a program of physical exercises and training for the use of firearms. Service hours may vary depending on service needs, and it is common for members of these units to work a high number of overtime hours, even at night. Exposure to acts of violence is inherent to professional commitment. The health monitoring and promotion project for the police officers was authorized by the Italian Ministry of the Interior in agreement with all the workers’ trade unions.

The control of the workers’ mental health status (e.g., stress) was delegated to a university professor (N.M.) external to the administration, to ensure that any findings of stressful conditions would not have a negative impact on the careers or earning capacity of the workers. Workers participated in the different phases of the program at high rates. The study protocol was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore of Rome, Italy (approval n. 285, 16 July 2020), and conducted in accordance with the Declaration of Helsinki. All participants provided their written informed consent to participate in the study. Since only two female workers were recruited, they were excluded from the analyses reported in this study for statistical reasons. Current smokers as well as subjects with periodontal problems were excluded. Sociodemographic and work-related characteristics, including age, education, marital status, presence of offspring, type of housing, military rank, work experience, and past medical history, were collected through interviews.

Stress assessment

Accurate measurement of chronic occupational stress requires addressing several challenges. Stress measured in an occupational cohort cannot be compared with that of the general population, which is not exposed to occupational stressors. Consequently, occupational studies require internal control. The subjective nature of the SR makes it difficult to establish the cut-off score of questionnaires.

Furthermore, a worker's perception of occupational stress is inevitably influenced by life events and may therefore vary over time in response to such unpredictable events. To minimize these problems, workers from the Genoa cohort were compared with themselves, examining the distribution of police officers' responses to a homogeneous occupational stressor, namely public order tasks. Furthermore, the questionnaires were administered multiple times each year of observation, so that any external event could affect only the measurement closest in time, with little effect on the overall classification.

Work-related stress was measured using two complementary models to capture the different aspects of the interactions between professional conditions and individuals that arise in relation to different operational situations[81]. The Italian versions[82] of the short forms of the Karasek Demand/Control questionnaire[83] and the Effort/Reward Imbalance questionnaire[18] were administered repeatedly to all cohort members.

The distribution of scores for each questionnaire administered during the first decade of observation was divided into quartiles, and each worker was assigned a score from 1 to 4 corresponding to their quartile. The sum of the scores yields an ordinal measure, “Stress Response” (SR), with values ranging from 1 to 40. Higher SR values indicate a greater response to homogeneous stressors in public order control[29].

Leaving the Mobile unit cohort can occur for supervening age limits and retirement, but more frequently, the dropout can occur due to the reporting of health problems that lead to moving to other forms of state service. During the observation period, the percentage of workers in the highest SR who continued to serve in the unit decreased more than that of the most resilient workers. Moreover, the subdivision of workers according to their level of resilience allowed us to verify that those belonging to the extreme groups differed in terms of personality traits[84], levels of anxiety and depression[22], absenteeism rates[85], quantity and quality of sleep[86], and metabolic syndrome incidence[23].

Saliva collection and nucleic acid extraction

Saliva samples were collected from January to March 2022 from 113 subjects using the Isohelix GeneFiX™ DNA/RNA Saliva collectors kit (Isohelix), following the manufacturer’s instructions. Participants were asked to refrain from eating for at least 1 hour prior to oral specimen collection. Saliva aliquots (1 mL) were stored at − 80°C until RNA and DNA extraction. Total RNA from saliva samples was extracted using the Maxwell® RSC miRNA Tissue kit (Promega) following the manufacturer’s instructions. Initially, 400 µL of saliva samples were added to chilled 200 µL of 1-Thioglycerol per 1 mL of Homogenization Solution, following the instructions of the manufacturer. At the end of the automated extraction, RNA samples were eluted in 60 µL of water. RNA concentration was measured with a Qubit fluorometer using Qubit microRNA assay (Thermofisher). DNA was extracted from 500 µL of saliva with the Maxwell®RSC Stabilized Saliva DNA kit (Promega) according to the manufacturer’s instructions. At the end of the automated extraction, DNA was eluted in 60 µL of water. The DNA quantification was performed with a Qubit fluorometer (Qubit DNA HS Assay Kit; Invitrogen).

Library preparation for small RNA sequencing

Small RNA sequencing (small RNA-seq) libraries were prepared from RNA extracted from saliva following a protocol for library prep described in[87,88]. Briefly, the NEBNext Multiplex Small RNA Library Prep for Illumina kit (New England Biolabs) was used to convert small RNA transcripts into barcoded complementary DNA (cDNA) libraries. For each library, 100 ng of RNA was processed as starting material. Each library was prepared with a unique indexed primer. Multiplex adapter ligations, RT primer hybridization, RT reaction, and PCR amplification were performed according to the manufacturer's protocol. After PCR amplification, the cDNA constructs were purified using the Monarch PCR & DNA cleanup Kit (New England Biolabs), following the modifications suggested in the NEBNext Multiplex Small RNA Library Prep for Illumina protocol. Final libraries were loaded on the Bioanalyzer 2100 (Agilent Technologies) using the DNA High Sensitivity Kit (Agilent Technologies) according to the manufacturer's protocol. Libraries were pooled together (in 35-plex or 40-plex) and further purified with a gel size selection. A final Bioanalyzer 2100 run using the High Sensitivity DNA Kit (Agilent Technologies) was performed to assess DNA library quality with respect to size, purity, and concentration.

The obtained libraries were subjected to the Illumina sequencing pipeline on an Illumina NextSeq500 sequencer (Illumina). Raw and processed sequencing data were deposited on Gene Expression Omnibus (GEO) with the identifier GSE285846.

Small RNA-Seq data analysis

Small RNA-seq analyses were performed using a Docker-based pipeline to ensure computational reproducibility[89]. Specifically, trimmed reads were mapped against a curated reference of human miRNAs based on miRBase v22.1. BWA algorithm v0.7.12.12 was used for read alignments on miRNA hairpin sequences. Mature miRNA levels were quantified as previously described[89]. In the case of mature miRNAs with identical sequences, the associated read counts were summed.

Differential expression analysis was performed using the DESeq2 R package (v1.40.2) with the likelihood ratio test, adjusting for age and sequencing pool. A miRNA was considered differentially expressed (DE miRNA) if associated with an adjusted $p < 0.05$ and a median number of reads > 10 in at least one study group.

Functional enrichment analysis was performed using RBiomirGS v0.2.19 with default settings and the IWLS method for the rbiomirgs_logistic function. Only validated miRNA-target interactions from miRTarBase 7.0 and miRecord were considered. The enrichment analysis was performed using the gene set libraries (2.cp.reactome.v2023.2, c5.go.bp.v2023.2, c2.cgp.v2023.2) from MSigDB (v2023.2.Hs). A term was considered enriched if associated with an adjusted $p < 0.05$ and at least two target genes. The input for the analysis was the average log2 fold change and the Fisher-combined adjusted P value from the differential expression analyses.

Library preparation for shotgun metagenomic sequencing

Sequencing libraries were prepared starting from 24 ng of DNA using the Illumina® DNA Prep (M) Tagmentation kit (Illumina), following the manufacturer's guidelines and as described in[90]. The library pool was subjected to a cleaning step with 0.7x Agencourt AMPure XP beads as described in[91]. Samples were sequenced on a NovaSeq 6000 flow cell (Illumina) at the Italian Institute for Genomic Medicine (IIGM) sequencing facility. Sequencing reads were deposited on SRA with the accession PRJNA1327569.

Shotgun metagenomics data analysis

The read preprocessing (adapter trimming and removal of low-quality reads), the alignment on PhiX control, and the human genome (hg38) were performed as described in the study by[90,91], using the pipeline available at https://github.com/SegataLab/preprocessing. The preprocessing steps include: for: i) removal of low-quality reads (quality Q < 20), too short fragments (length < 75 bp), and reads with two or more ambiguous nucleotides; ii) host and contaminant DNA removal using Bowtie 2.77 (--sensitive-local) for filtering the phiX174 Illumina spike-in and human-aligned reads (hg38 assembly); iii) creation of paired forward and reverse and unpaired reads output files.

Taxonomic profiling was performed with MetaPhlAn 4.1 with the "--statq 0.1" and the ChocoPhlAn vJun23 database[92]. Microbial pathway abundances were estimated using HUMAnN 3.9[93], applied with default settings and using the full UniRef90 as the reference database. Low-abundant species were removed using the *nearZeroVar* function of the caret R package. Diversity metrics (richness, Inverse Simpson index, Shannon index, and evenness) were computed using the *vegan* R package v2.5-6.1. Differential abundance analysis was performed with SIAMCAT v2.12[94]. The analysis was performed on all taxonomic levels, including the Species-level Genomic Bins (SGBs) specifically profiled using MetaPhlAn 4.1, by removing the low-abundant taxa (*nearZeroVar* function of caret R package).

Statistical and computational analyses

All statistical analyses and graphical representations were performed using R (version 4.5). Statistical analysis between continuous variables was performed using the Wilcoxon rank sum test or Kruskal-Wallis test. Statistical analysis between categorical variables was performed using the chi-square test. Correlation analyses were performed using Spearman's rank correlation.

Significant microbial pathways were selected using the Wilcoxon rank-sum test, based on the global pathway score. Only tests with a Benjamini-Hochberg-adjusted p-value < 0.1 were considered. Then, individual microbial contributions (genus-level) were calculated using the same method, considering only tests with $p < 0.05$.

DIABLO module of MixOmics v.6.32[95] was used to integrate identified relevant miRNAs and taxonomic profiles, levels, and HUMAnN microbial pathways. The analysis was conducted using the projection to latent structures (PLS) method, which applies sparse multiblock partial least squares discriminant

analysis for simultaneous integration and variable selection. For the analysis, the first two components were considered, and only significant correlations were selected.

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Università Cattolica del Sacro Cuore of Rome, Italy (approval n. 285, 16 July 2020), and was conducted in accordance with the ethical standards of the Declaration of Helsinki. All participants provided their written informed consent to participate in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed in the current study are available in GEO and SRA under the accession numbers GSE285846 (small RNA-Seq) and PRJNA1327569 (shotgun metagenomics).

Competing interests

The authors declare no competing interests.

Authors' contributions

SG: conceptualization, methodology, project administration, supervision; NM: conceptualization, methodology, supervision; BP and ST: methodology, investigation; GF and AC: formal analysis, data curation, visualization; FC: supervision; EG: investigation, writing - original draft; AN: resources, funding acquisition, supervision. All authors read and approved the final manuscript.

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Table

Table 1. Sociodemographic and occupational variables of the study population stratified for stress response (SR) categories.

Variables	Low SR	Intermediate SR	High SR	p[3]
	N = 31	N = 55	N = 27	
Age, years [1]	45 (±5)	45 (±5)	42 (±5)	**0.04**
BMI[1]	26.1 (±3.9)	26.8 (±3.6)	26.6 (±3.0)	0.40
Education[2]				0.40
University degree	1 (3.2%)	2 (3.6%)	0 (0.0%)	
High school	20 (64.5%)	41 (74.6%)	23 (85.0%)	
Middle school	10 (32.3%)	12 (21.8%)	4 (15.0%)	
Marital status[2]				0.60
Unmarried	15 (48.4%)	23 (41.8%)	16 (59.3%)	
Married	15 (48.4%)	28 (50.9%)	9 (33.3%)	
Separated	1 (3.2%)	4 (7.3%)	2 (7.4%)	
Children[2]				0.30
N	17 (55.0%)	27 (49.0%)	18 (67.0%)	
Y	14 (45.0%)	28 (51.0%)	9 (33.0%)	
Settled down[2]				0.12
N	21 (68.0%)	36 (65.0%)	12 (44.0%)	
Y	10 (32.0%)	19 (35.0%)	15 (56.0%)	

[1]Mean (±standard deviation); [2]n (%); [3]Kruskal-Wallis test; Fisher's exact test; Pearson's Chi-squared test; N: no; Y: yes.

Figures

Figure 1

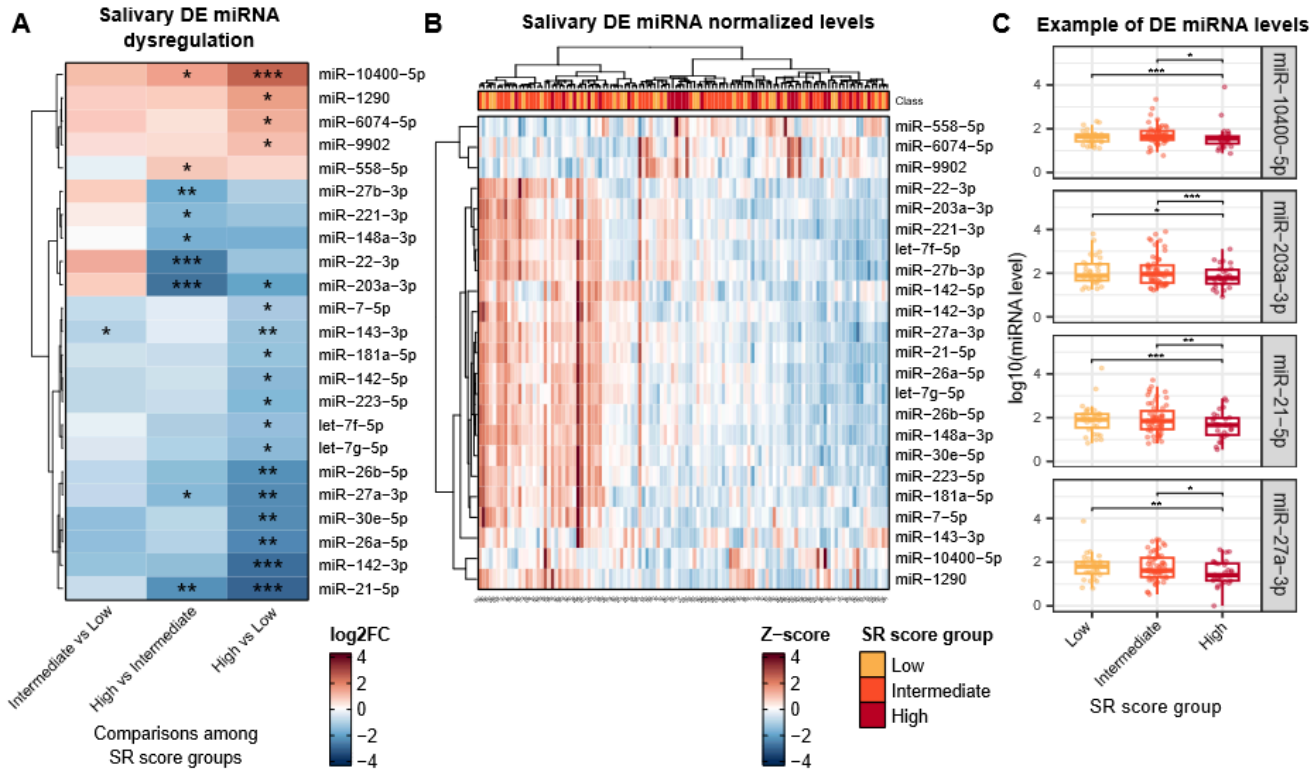


Figure 1

Analysis of dysregulated salivary miRNA profiles. Heatmap of the log2FC (**A**) and Z-score (**B**) of the DE miRNAs observed in at least one comparison. In (**B**), for each participant, the SR (Stress Response) score level is reported. Adjusted p-value from DESeq2 analysis: ***adj. p < 0.001; **adj. p < 0.01; *adj. p < 0.05. **C**) Boxplot of four miRNAs with levels significantly different in at least two comparisons among subjects stratified according to SR scores. ***adj. p < 0.001; **adj. p < 0.01, *adj. p < 0.05.

Figure 2

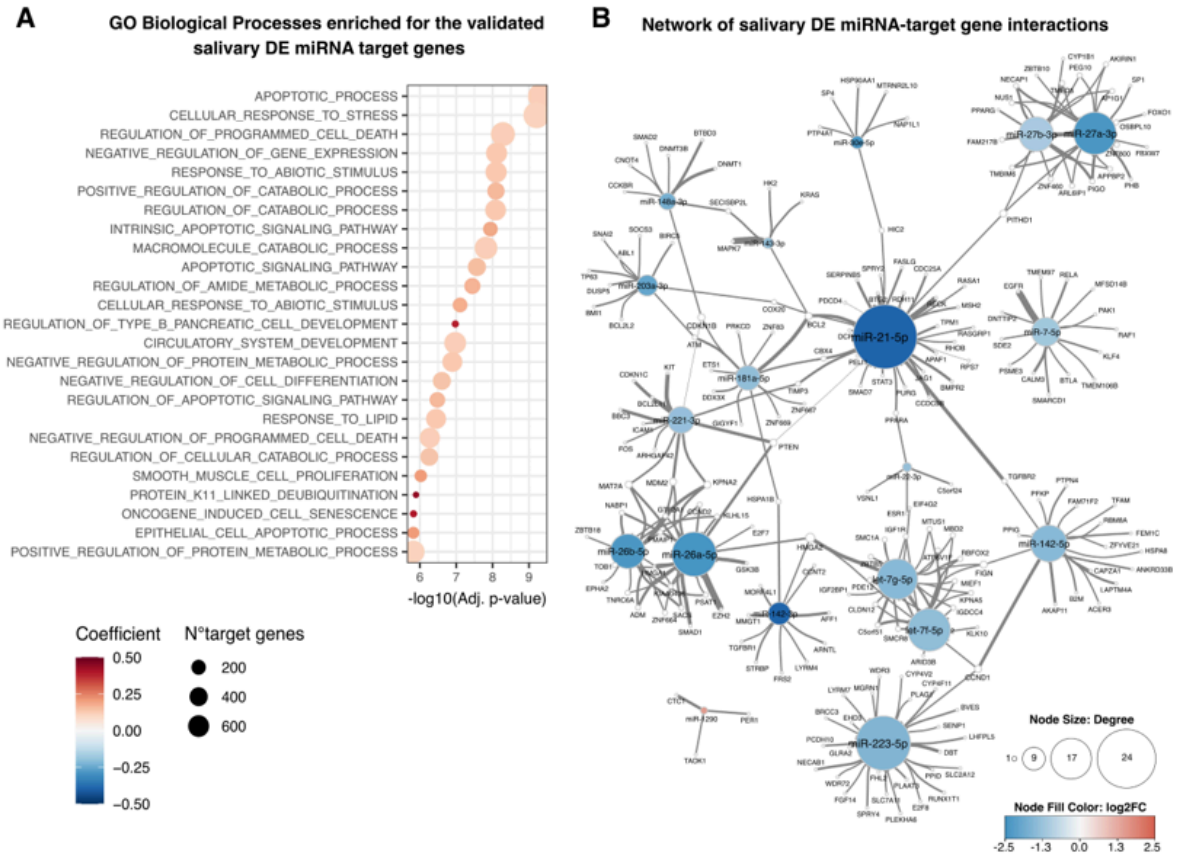


Figure 2

Functional target enrichment analysis of dysregulated salivary miRNAs. A) Functional enrichment analysis results from RBiomirGS analysis. The dot size is proportional to the number of target genes, while the color code represents the pathway activation coefficient. Red-colored coefficients represent pathways predicted to be activated based on targeting miRNA expression changes. **B**) Network representation of the miRNA-target interaction supported by at least four independent publications. The node size is proportional to the total node degree, while the color-code reflects the expression change (log2FC) computed between the low- and the high-SR groups. Edge tightness is proportional to the number of supporting evidence.

Figure 3

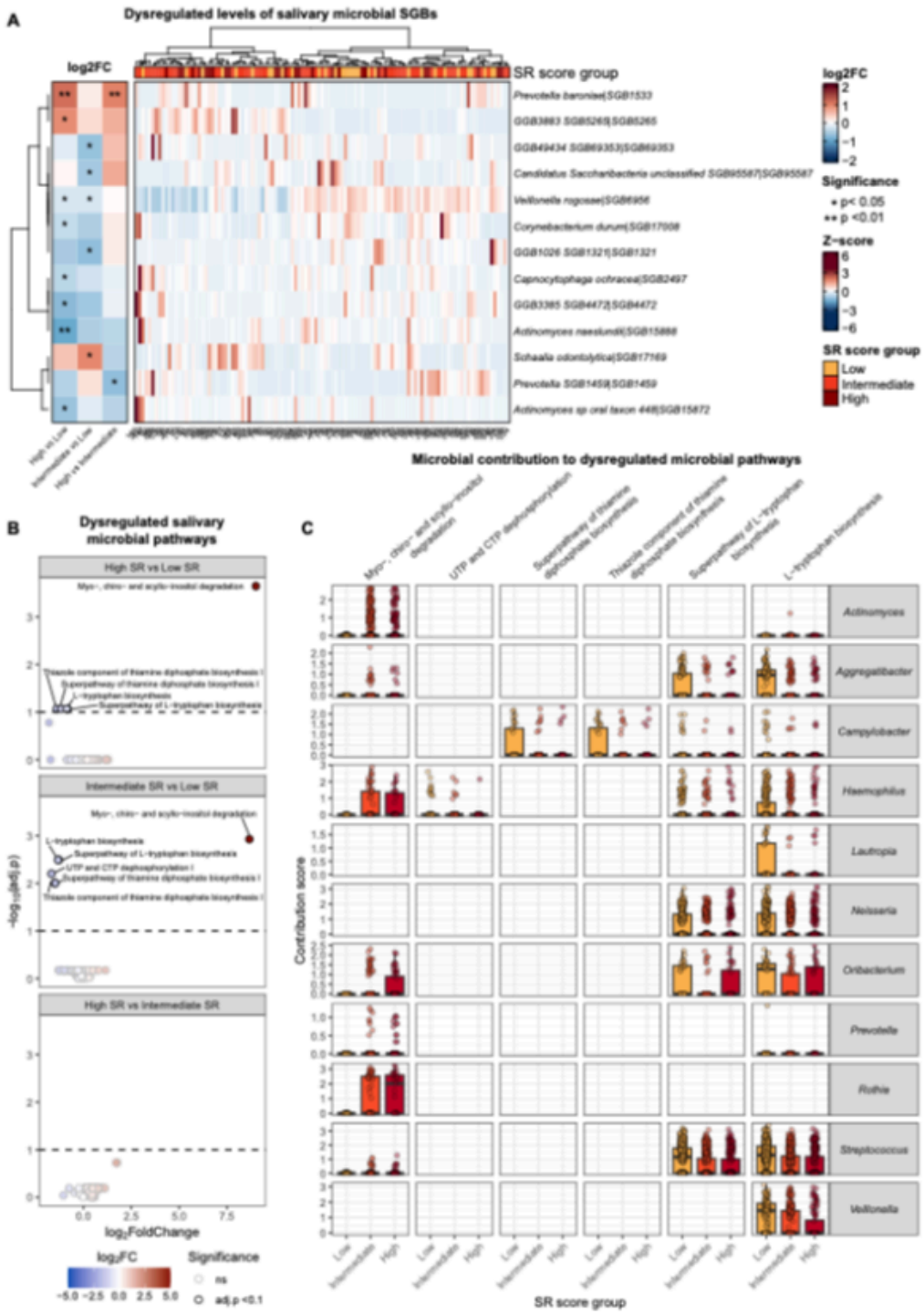


Figure 3

Analysis of salivary microbial communities. A) Heatmap reporting the log2FC (left) and Z-score (right) of differentially abundant SGBs in at least one comparison. For each subject, the SR score is reported (*p < 0.05; **p < 0.01). **B**) Volcano plots reporting the results of the microbial pathways analysis among SR groups. On the X-axis, the log2FC is reported, while on the Y-axis, the significance (adj. p-value) is shown. The black dashed line indicates the adj. p-value threshold of 0.1, and the black dot border highlights the

significant ones. **C**) Boxplots reporting the significant contribution from different microbial genera to each pathway (contribution score, Y-axis). The box and dot color reflect the SR group.

Figure 4

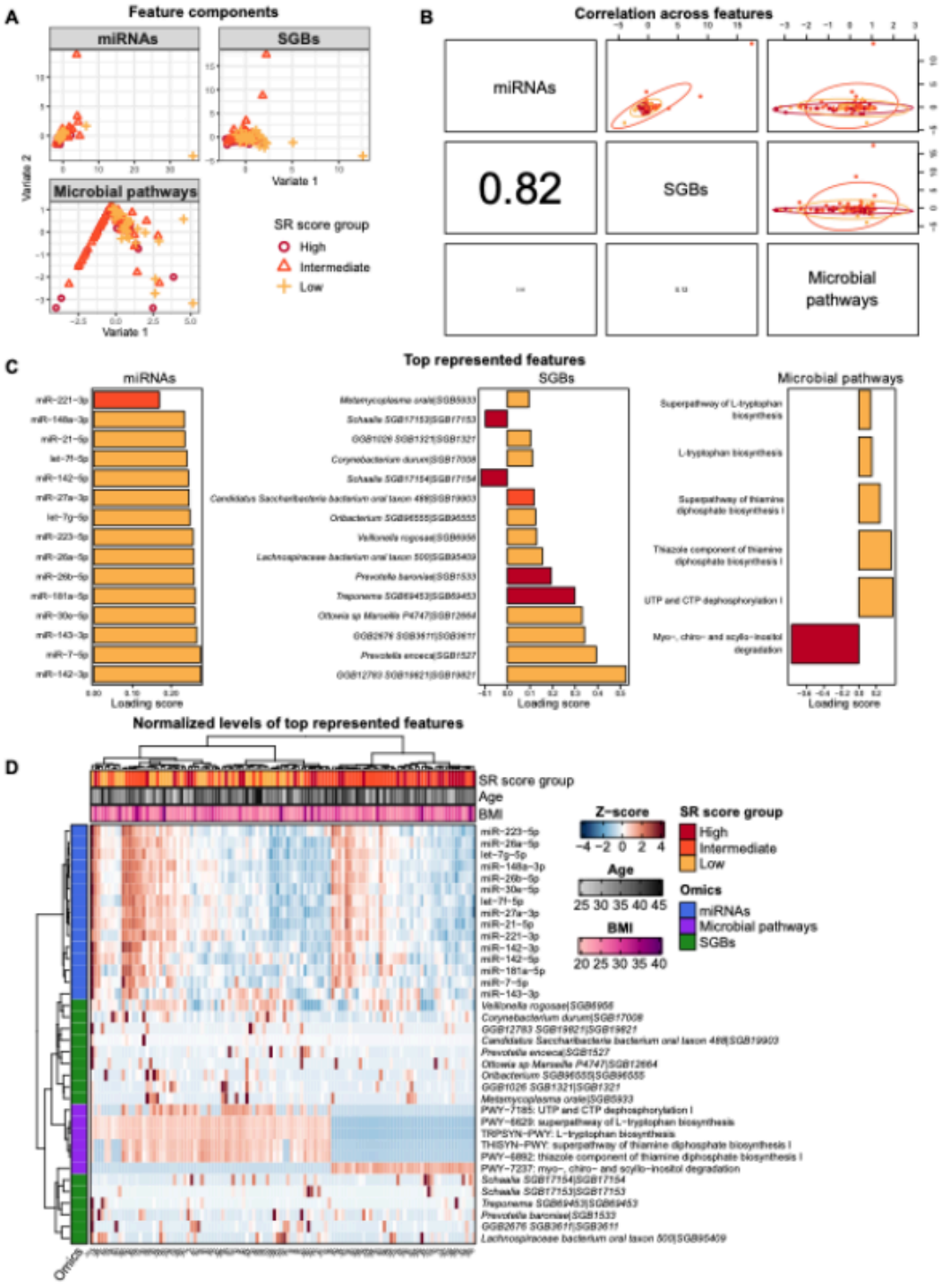


Figure 4

Integrative analysis of salivary miRNA and microbial profiles. A) PLS plot reporting Variate 1 and Variate 2, which represent the components capturing the highest covariance between omics features

and SR groups. **B**) Correlation plot showing the relationships between variates across the different omics datasets. **C**) Bar plots reporting the top-represented features in each omics dataset. The X-axis reports the loading score, indicating the weight of each feature's contribution, while the bar's color reflects the SR group. **D**) Heatmap reporting the unsupervised clustering based on Z-scores of the top correlated features. The single omics are highlighted with a specific color, while a different color key highlights the subject's SR group. For each subject, the age and BMI value are also reported.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1SalivarymiRNAanalyses.xlsx
- SupplementaryTable2MicrobialspeciesandPathways.xlsx
- FigureS1.pdf