

TITLE: miRNA-mediated regulation of Mucin-type *O*-Glycosylation pathway: a putative mechanism of salivary gland dysfunction in Sjögren's syndrome.

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Running title: **pSS:miRNA and Mucin-*O*-Glycosylation**

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ABSTRACT

Objectives: To investigate microRNA (miRNA)-potentially implicated in primary Sjögren's syndrome (pSS)-related salivary hypofunction in labial salivary glands and to explore miRNA-mediated mechanisms underlying oral dryness and altered rheology focusing on mucin *O*-glycosylation pathway.

Methods: We performed miRNA expression profiling in minor salivary gland samples of pSS patients presenting a different impairment in their unstimulated salivary flow rate (USFR). A computational *in silico* analysis was performed to identify genes and pathways that might be modulated by the deregulated miRNAs that we had identified. To confirm *in silico* analysis, expression levels of genes encoding for glycosyltransferases and glycan-processing enzymes were investigated by using Human Glycosylation- RT² Profiler™ PCR Array.

Results: Among 754 miRNA analyzed, we identified 126 miRNAs that were significantly de-regulated in pSS compared to controls, with a trend that was inversely proportional with the impairment of salivary flow rates. An *in silico* approach pinpointed that several upregulated miRNAs in pSS patients target important genes in the mucin *O*-glycosylation. We confirmed this prediction by qRT-PCR, highlighting the downregulation of some glycosyltransferase and glycosidase genes in pSS samples compared to controls, such as GALNT1, responsible for mucin-7 glycosylation.

Conclusion: Collectively, our data suggest that the expression of different predicted miRNA-target genes in the mucin type *O*-glycan biosynthesis pathway is altered in pSS patients with low salivary flow and that miRNome could influence the glycosidases expression levels and consequently the rheology in pSS.

Keywords: Sjögren's syndrome, MiRNA, pathogenesis, epigenetics

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a complex, heterogeneous and disabling disorder primarily affecting the exocrine glands but potentially involving any other organ and systems (1-5). The hallmark of the disease is the lymphocytic infiltration and hypofunction of salivary and lachrymal glands that leads progressively to the typical oral and ocular dryness (5-8).

Pathogenetic mechanisms underlying salivary hypofunction in pSS have been only partially elucidated at the moment, especially due to the fact that the correlation between salivary gland inflammation and dysfunction has appeared as relatively scarce (9).

Recently, several "omics" studies have been encouraged, ultimately aimed at better clarifying pSS salivary impairment with the general hypothesis that epigenetic mechanisms may influence salivary flow, protein expression and saliva properties.

From this perspective, a number of differently expressed miRNAs have been described in minor salivary gland biopsies of patients with pSS and associated with either salivary gland focus score or salivary flow decrease; in parallel, proteomic analysis of saliva has highlighted several changes in the protein composition of saliva in pSS potentially associated to the alteration of salivary secretion (10-13). In particular, several quantitative differences have been described in the salivary proteome of pSS patients with respect to healthy volunteers, with a significant increase of proteins related to inflammation and immune response and a decrease of normal constituents of saliva. Moreover, qualitative differences in protein post-translational modifications have been highlighted as well, especially in the mucin *O*-glycosylation processes (14-18). Mechanistic studies suggested that changes in the glycosylation of mucins rather than the proteins themselves may occur as the direct result of local inflammation induced by proinflammatory mediators, such as interleukin-1. (19, 20). Overall, the altered expression of the mucins in pSS saliva and tears has appeared as affecting negatively the

rheology of the salivary flow and the tears, ultimately contributing to the oral and ocular dryness (19, 20).

In this exploratory descriptive study, therefore, we aimed at investigating whether a miRNA-mediated mechanism could be involved in salivary hypofunction, influencing salivary proteome and mucin alterations. More specifically, we first identified miRNA differentially expressed in pSS patients with decreased salivary flow with respect to pSS patients with normal salivary flow and to healthy controls. Subsequently, we examined the pathways targeted by the putative deregulated miRNAs, including the mucin-type O-glycosylation pathway in order to ultimately evaluate the impact of epigenetics on salivary rheology.

MATERIALS AND METHODS

Patient and sample collection

We selected in this study minor salivary gland biopsies (MSGBs) from patients newly diagnosed with pSS (AECG criteria 2002) (21). All the patients underwent a complete work-up for the diagnosis of the disease including sialometry. We defined patients as having a low salivary flow (LF) if their unstimulated salivary flow rate (USFR) was lower or equal than 1.5 ml/15 minutes and as having a preserved salivary flow (HF) if their USFR was higher than 1.5 ml/15 minutes. For the purposes of this study, in order to reduce the heterogeneity derived from different degree of inflammation in the minor salivary gland samples, we excluded pSS patients with a low or moderate infiltrate, and we retrieved only biopsies with a focus score ≥ 3 (22). Subsequently, we subdivided our samples in two groups: group (a) biopsies from patients having a HF and group (b) biopsies from patients having a LF. Controls (CTR) were represented by patients undergoing a MSGB under the suspicion of pSS, who at the end of the work-up were not diagnosed with pSS and that at the MSGB histology presented a mild aspecific inflammation. All patients gave written informed consent for all procedures, which were

carried out with local ethics committee approval (Comitato per la Sperimentazione Clinica dei Medicinali, University of Pisa, prot N°3062/2010).

Minor salivary gland biopsy: Minor salivary gland samples, formalin-fixed, paraffin embedded, sectioned and haematoxylin and eosin stained, were evaluated by the same pathologist. If a diagnosis of focal lymphocytic sialadenitis was made, the focus score (FS) was then determined according to the method of Greenspan et al. (23). A focus was defined as an aggregate of ≥ 50 mononuclear cells (mostly lymphocytes). The FS was reported as the number of foci per 4 mm^2 of tissue, up to a maximum of 12 foci. Only samples with a $\text{FS} \geq 3$ were selected for the study in order to select samples homogeneous in their inflammatory infiltrate composition and to minimize the impact of inflammation on salivary gland epithelium function.

Sialometry: According to the AECG 2002 criteria (21) patients were asked to sit quietly, without talking or chewing, and spit any saliva that accumulates in the floor of their mouth into a pre-weighed tube for a total of 15 minutes.

RNA extraction

Total RNA was purified with the miRNeasy mini kit (Qiagen, CA), following the manufacturer's recommendations. RNA concentration was evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

TaqMan low-density arrays (TLDA) for miRNAs profiling

MicroRNA profiling of samples was done with TaqMan Array Human MicroRNA panels A and B (Life Technologies, Thermo Fisher Scientific) to analyze 754 human miRNAs. Reverse transcription

and pre-amplification were done following the manufacturer's instructions (Life Technologies, Thermo Fisher Scientific). QRT-PCR was performed with the Applied Biosystems 7900 HT Real-Time PCR system. For each miRNA, the expression level was determined by the equation $2^{-\Delta\Delta CT}$ (24). U6 snRNA (001973) was used as control to normalize data. Gene Expression Suite software (v1.0.4) and Data Assist software (v 3.01) (Life Technologies, Thermo Fisher Scientific) were further used to process the array data.

In silico analysis

A computational analysis was performed to identify genes and pathways that might be modulated by the deregulated miRNAs that we identified. For the in silico analysis, we used DIANA-miRPath v.3 (25) to identify potential miRNA target genes and pathways (as settings, we used microT-CDS for target prediction with a threshold of 0.8). Results were visualized with a p value threshold of 0.05 using the option "genes union", according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). In addition, we used Cytoscape (26) for visualizing selected miRNAs target genes interactions network.

Analysis of glycosylation enzymes genes

The cDNA for each RNA sample was obtained using RT² First Strand kit (SABiosciences Corporation, Qiagen, Frederick, MD), according to the manufacturer's instructions. The expression levels of glycosyltransferases and glycosidases were analyzed using RT² Profiler™ PCR Array - Human Glycosylation (PAHS-046ZC; SABiosciences Corporation, Qiagen), following the manufacturer's instructions. An Applied Biosystems StepOne Plus PCR System (Life Technologies, Thermo Fisher Scientific) was used for measurements. The PCR array data were analyzed by RT² Profiler PCR Data Analysis software (SABiosciences Corporation; <http://sabiosciences.com/pcrarraydataanalysis.php>).

Relative quantification for each gene was assessed by $2^{-\Delta\Delta CT}$ calculation for each mRNA. All test samples were run in duplicate and template-negative reactions served as controls. Expression of each gene was classified as 'high' or 'low', based on the level of expression after grouping patients.

Statistical analysis

QRT-PCR data processing and analysis were conducted using tools from RQ-manager (v1.2, Life Technologies, Thermo Fisher Scientific), Expression Suite software (v1.0.4, Life Technologies, Thermo Fisher Scientific), Microsoft Excel and Prism GraphPad V5.0d software (GraphPad Software, CA). In addition, real-time PCR array data were analyzed using the RT² Profiler PCR Array Data Analysis program (SABioscience, Qiagen; <http://sabiosciences.com/pcrarraydataanalysis.php>).

In order to summarize the most relevant features of pSS patients, descriptive statistics were used. In particular, for categorical variables we reported the distribution of absolute frequencies, percentages and cumulative, while for continuous variables the mean, standard error.

To evaluate differences between pSS samples, analysis of variance (ANOVA) was used. Bartlett's test was also applied in order to assess the homoscedasticity assumption.

p-values <0.05 were considered to be statistically significant. In the figures, * and ** indicate statistical significance at p <0.05 and 0.01, respectively. All statistical analyses were performed using STATA 13.1 and R 3.3.

To visualize principal component analysis (PCA), the R functions "prcomp" and "plot" were used (27, 28).

RESULTS

Cellular miRNome significantly changes in pSS patients.

To study the role of miRNA expression profile (miRNome) in salivary glands of pSS, we analyzed miRNA expression by qRT-PCR arrays in human MSGB from non-SS controls (n=5), pSS patients with HF (n=5), and with LF (n=5). Controls (i.e non-SS controls) were represented by 5 women aged 54.9 ± 17 years with sicca symptoms, negative anti-Ro/SSA and a mild aspecific sialoadenitis in the MSGBs. Regarding pSS patients, we chose to investigate the miRNome in pSS with a FS ≥ 3 , but different salivary flow phenotypes, in order to reduce the variability of B-cell infiltrates that might bring to misleading results in the salivary flow mechanism comprehension. No statistically significant difference in the FS was observed between HF and LF pSS patients. Supplementary Table 1 summarizes the clinical and serological features of the patients enrolled in the study. As shown in Figure 1 A, we found that the expression levels of several miRNAs have a very specific pattern and the samples strongly cluster following the salivary flow stratification and with clinical characteristics.

To evaluate if the TLDA results could separate controls, HF and LF samples, an unsupervised PCA using the 126 significant differentially expressed miRNAs in pSS samples was performed (Figure 1 B). PCA showed that LF and control samples clustered into two separate populations, indicating that the two groups have a very different miRNA expression profile, while miRNA expression levels in HF are intermediate between controls and LF samples, corroborating the heat map diagram results.

Among 754 human miRNAs on the arrays (panels A and B), our analysis displayed a unique pattern of miRNA expression, highlighted by a marked over-expression of members of let-7, miR-30, miR-17/92, miR-200 microRNA families, in pSS samples relative to controls. Interestingly, the over-expression in pSS shows a trend that is inversely proportional with the impairment of salivary flow rates (Supplementary Figure 1).

We selected 126 microRNAs that were significantly de-regulated in pSS compared to control (Supplementary Tables 2). Among these 126 microRNAs, one hundred showed a very interesting expression pattern, with an over-expression inversely proportional to the salivary flow rate, suggesting

a global expression change in the miRNome of impaired salivary glands. In Figure 2 we show 6 representative graphs in which is represented the expression level of 4 up-regulated miRNAs (hsa-miR-18b, hsa-miR-20a, hsa-miR-106a, hsa-miR-146b), with an inversely proportional pattern to the salivary flow rate and 2 down-regulated (hsa-miR-635 and hsa-miR-372) with a directly proportional pattern to the salivary flow rate.

MiRNA target prediction and pathway enrichment analysis

To identify potential pathways which could be targeted by the most 100 deregulated miRNAs in the pSS samples (fold change ≥ 2 ; $p < 0.05$), we performed a computational analysis using DIANA-miRPath software (<http://snf-515788.vm.okeanos.grnet.gr/>).

KEGG pathways that theoretically might be affected by the upregulated miRNAs are reported in Figure 3 A, and among which the most significant ($p = 2.98e-13$) was “Mucin type O-Glycan biosynthesis” pathway.

We reported the list of the deregulated miRNA used for the bioinformatics analysis and of the predicted target genes of the Pathway 'Mucin type O-Glycan biosynthesis' in Supplementary Table 2.

As glycosylated mucins are important for saliva rheological properties (19, 29) and to confirm *in silico* analysis, we decided to investigate glycosyltransferase and glycosidase expression level changes in pSS patients and to evaluate the possible correlation with salivary flow.

Human Glycosylation profiling analysis in pSS patients

A total of 84 genes encoding enzymes that add glycans to proteins or remove glycans from glycoproteins were analysed using Human Glycosylation- RT² Profiler™ PCR Array in 4 healthy volunteers, 4 pSS patients with high salivary flow (HF) and 4 with low salivary flow (LF). The samples

used in miRNA and gene expression arrays were the same. The array results for the three groups are reported in Supplementary Table 3.

Hierarchical clustering showed systematic variations in the gene expression among the different groups, confirming that pSS samples (HF and LF) had similar gene expression profile, and both were different from controls (Figure 3 B).

As shown in volcano plots (Figures 3 C and D), using a threshold value of 2-fold expression change and $p < 0.05$, we found a gene expression deregulation of some glycosyltransferases and glycosidases.

In particular, compared to controls, one gene was significantly overexpressed (ST8SIA4) and 4 were downregulated (B4GALT2, B4GALT3, GALNT1, GALNT16) in both pSS groups (HF and LF) (Figures 3 C and D).

Other few genes were significantly downregulated in HF or LF respect to the controls, as reported in Supplementary Table 3. More specifically, we highlighted a trend for the expression of other genes, such as EDEM2, GALNT4, GLB1, which were highly downregulated in LF ($p < 0.05$) and less in HF, respect to the controls.

Collectively, these data suggest that different predicted miRNA-target genes in the mucin type *O*-glycan biosynthesis pathway were altered and that miRNome could influence the glycosidase expression levels and consequently the glycosylation of mucins (Figure 4).

Discussion

Pathogenetic mechanisms responsible of salivary hypofunction in pSS have been object of various studies, especially because the correlation between salivary gland inflammation and dysfunction has still not been completely clarified.

In the present exploratory study we investigated miRNAs that might be implicated in pSS salivary hypofunction and altered rheology in pSS by array analysis of MSGBs from pSS patients with HF or

LF, as well as non-SS sicca controls. We identified 126 de-regulated microRNAs that were inversely associated with the impairment of salivary flow rates. Either overexpressed or downregulated, these microRNAs followed perfectly the salivary flow rates in a proportional way. The plus/minus microRNA presence following the plus/minus salivary production, strongly suggested a direct role of the global miRNome of impaired salivary glands.

In addition, among 754 human miRNAs investigated, we found a marked over-expression of miR-30, miR-17/92, miR-200 and miR-let-7 family members, in pSS with respect to controls. These miRNAs families have been described as involved in several physiological and pathological processes including oncogenesis (30),(31, 32),(33) heart development (34), lung development (35), repression of cancer stem cell differentiation, modulation of cell division and apoptosis (36) and cell differentiation and proliferation (37). Gourzi VC et al. (38) found that the levels of miR200b-3p were up-regulated in SGECS of SS patients compared to sicca-complaining controls. The MSG levels of let7b, were correlated positively with Ro52/TRIM21-mRNA. miR200b-3p was correlated negatively with Ro52/TRIM21 and Ro60/TROVE2 mRNAs in SGECS, respectively, whereas let7b and miR200b-5p were associated with La/SSB-mRNA. In PBMCs, let7b were correlated with Ro52/TRIM21, whereas let7b, were also associated with La/SSB-mRNA expression. Significantly lower miR200b-5p levels were expressed in SS patients with mucosa-associated lymphoid tissue (MALT) lymphoma compared to those without. In our study for the first time, these miRNAs families were associated with the impairment of salivary flow rates.

To assess the global impact of differentially expressed miRNAs in pSS samples, an *in silico* approach was used to identify the major signaling pathways that could be most likely affected by the co-expressed miRNAs.

We found that several upregulated miRNAs in pSS patients predicted to target genes implicated in mucin type-O glycosylation. Indeed, Real-time RT-PCR analysis confirmed the down-regulation of

some glycosyltransferase and glycosidase genes in pSS samples compared to controls, suggesting that the altered salivary function and glycosylation in pSS may be regulated by miRNAs.

In particular we found that GALNT1, the predicted target of many upregulated miRNAs (miR-24-2-5p, miR-23a, miR-590-3p, miR-30b, miR-335-3p, miR-539, miR-323-3p, miR-192-3p, let-7g, let-7d, let-7e, miR-7 1-3p, miR-543, let-7c), was downregulated in pSS patients.

GALNT1 is responsible for glycosylation of mucin-7 (MUC7) (39-41), a large, very heavily *O*-glycosylated salivary protein (150 kDa), containing central mucin domains, rich in serine and threonine residues that covalently bind the *O*-glycosylation important for salivary viscoelastic behavior and lubrication of the oral mucosa (42).

Notably, a dramatic reduction in MUC7 glycosylation was found in pSS patients and it was associated to oral dryness (16, 19, 29). Chaundury N.M.A, in particular, observed that a decrease in the extended, fucosylated core 2 disialylated structure on MUC7 was significantly associated with the perception of oral dryness in pSS patients (24).

Several other genes implicated in glycosylation were significantly different between SS and controls including GALNT2, GALNT4, B4GALT5, ST3GAL1, CIGALT1, CIGALT1CI and GCNT1. Few genes (i.e EDEM2, GALNT4 and GLB1) also, tend to be differently expressed in SS patients with low with respect to patients with high salivary flow rate. However, although the pattern of miRNA expression differs between SS patients with low or high salivary flow rate and associates with altered glycosylation pathway in array analyses, the expression of the major genes that are implicated in glycosylation did not support this difference.

The fact that we were not able to highlight a significant different expression in glycosylation genes between the two SS subgroups may be due, in our opinion, to different biological biases. Firstly, we know that the miRNA mechanism action is through either mRNA degradation or suppression of mRNA translation. In particular, regulation of glycoenzymes (themselves proteins) occurs at multiple steps,

including transcriptional, translational and post-translational levels (43). Although transcript (i.e. cellular mRNA) levels are often used as a key indicator of what glycogenes are being translated and the levels of biosynthetic enzymes and glycans that result, the relationship is not straightforward. However, changes in glycan structures upon perturbation do not correlate with changes in glycogene mRNA expression levels (43). Although part of this discrepancy may be due to difficulties with quantitatively measuring low-abundance glycogene transcript levels, the post-transcriptional regulation of glycogene expression may also control carbohydrate structures (44). We think that since the relationship miRNA-glycogenes mRNA is often non-linear, this may reflect the complexity of the system regulation, although there is in the SS samples a significant reduced glycogenes mRNA presence (confirming the altered glycosylation in pSS, as reported by other groups). We are aware that the sample size may reflect also this assay instability, but the correlation of a cellular pathway as the O-glycosylation pathway may be the link between the miRNAs profiling and the already well-known glycosylation decrease on mucins in SS patients.

Despite these limitations, our study may have several points of strength. First, our experiments are consistent with previous studies on miRNome profiling in pSS (45, 46). We substantiated previous findings on the de-regulation of a large number of microRNA in pSS MSGBs (10, 13, 45). In addition, the use of TaqMan low-density arrays (TLDA) for miRNAs profiling allowed us to describe a novel list of miRNA and miRNA families potentially implicated in the disease, thus broadening the scenario of epigenetics contribution in pSS pathogenesis. More remarkably, our set of descriptive observations indicated that miRNAs might be implicated in the de-regulated glycosylation and hypofunction of salivary glands in pSS. However, we are aware that our research has a number of limitations as well. Giving that our findings are based on descriptive and not functional studies, indeed larger functional investigation on MUC7 O-glycosylation should be undertaken in the next future to confirm the link between mi-RNA de-regulation, altered mucin glycosylation, and pSS-related hyposalivation.

Moreover, due to the restricted number of samples analyzed, data experiments require validation in a replication cohort to be generalized.

Nevertheless, our exploratory study still represents a first interesting attempt of how the concomitant use of complementary innovative “omics” techniques may be useful to foster the comprehension of complex pathogenetic mechanisms underlining different phenotypic subsets of pSS. From this point of view de-regulated mi-RNAs could represent not only biomarkers for distinct subsets of the disease but also putative targets for future therapies.

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FIGURE LEGENDS

Figure 1

A) Unsupervised hierarchical clustering and analysis of miRNA expression profiles in controls and pSS samples. Expression data of 784 miRNAs were obtained with Custom TaqMan[®] Array MicroRNA Cards (Life Technologies, Thermo Fisher Scientific), analyzed using RQ-manager (v1.2) and DataAssist (v3.01). The 126 deregulated miRNAs in pSS samples respect to controls are shown in the heat map. The distance measured is Pearson's Distance, and the clustering method is average linkage. Each column corresponds to the sample expression profile, and each row corresponds to a miRNA. The miRNA clustering tree is shown on the left. Branch lengths represent the degree of similarity between individual miRNAs. Red and black colours indicate relatively high and low expression, respectively.

B) Principal component analysis (PCA) was performed on all samples (n=5 for each group) and on the

top 126 microRNAs with $p \leq 0.05$). Normalized values ($\text{Log}_{10} 2^{-\Delta\Delta\text{CT}}$) were used for the analysis. Red = control group; green= HF group; blue=LF group. There is clear separation of LF and control groups, demonstrating a clear different miRNA expression profile.

Figure 2

Expression levels of 6 deregulated miRNAs in controls (CNT), HF and LF pSS patients. Values were normalized to U6 levels and expressed as the fold increase over controls. Data are shown as the mean \pm SD. * $p < 0.05$.

Figure 3.

Patients with primary Sjogren's syndrome (pSS) differentially express glycosylation enzymes respect to control group.

A) Top overrepresented KEGG pathways from DIANA-mirPath v3.0 analysis, based on the most 100 deregulated miRNAs in pSS samples (HF and LF). Significant pathways were determined using the union of predicted miRNA target genes.

B) Non-supervised hierarchical clustering based on the gene expression levels in control group, pSS patients high salivary flow (HF) and with low salivary flow (LF) ($n=4$ for each group). A total of 84 genes were analysed using RT² Profiler™ PCR Array - Human Glycosylation. The dendrogram shows the relationships among gene expression patterns: “red” indicates high relative expression, “green” low relative expression.

Volcano plots showing differentially expressed genes between HF vs control group, **C)**, and LF vs control group, **D)**. The volcano plot displays statistical significance versus fold-change on the y- and x-axes, respectively. 10 genes have significantly different expression in HF samples respect to controls ($p \leq 0.05$): 1 with fold change ≥ 2 , and 9 with fold change ≤ 0.5 . One gene was significantly upregulated

(fold change ≥ 2 ; $p \leq 0.05$) and 7 were downregulated (fold change ≤ 0.5 ; $p \leq 0.05$), when comparing the mRNA expression levels between LF and CNT samples.

Figure 4

Visualization of a portion of the miRNA-mRNA interaction based on correlation analysis of their expression in pSS samples. Negatively correlated miRNA-mRNA interactions were visualized as a network using Cytoscape. Diamond shapes represent miRNAs, while circle shapes represent miRNA targets (genes) of "Mucin type O-Glycan biosynthesis" pathway.

Downregulated genes in pSS patients are in orange, whereas the pink diamonds represent the up-regulated miRNAs.

Supplementary Figure 1

Deregulation expression patterns of miRNA families across different groups. CNT: control group; HF: pSS patients with high salivary flow; LF: pSS patients with low salivary flow (LF).

Supplementary Table 1: Demographics and clinic serological features of patients.

Supplementary Table 2: miRNA deregulated in controls, HF and LF groups (fold change ≥ 2 ; $p \leq 0.05$) and predicted target genes of the Pathway 'Mucin type O-Glycan biosynthesis'.

Supplementary Table 3: Fold regulation and p-values glycosyltransferases and glycosidases in HF and LF groups, comparing to controls.

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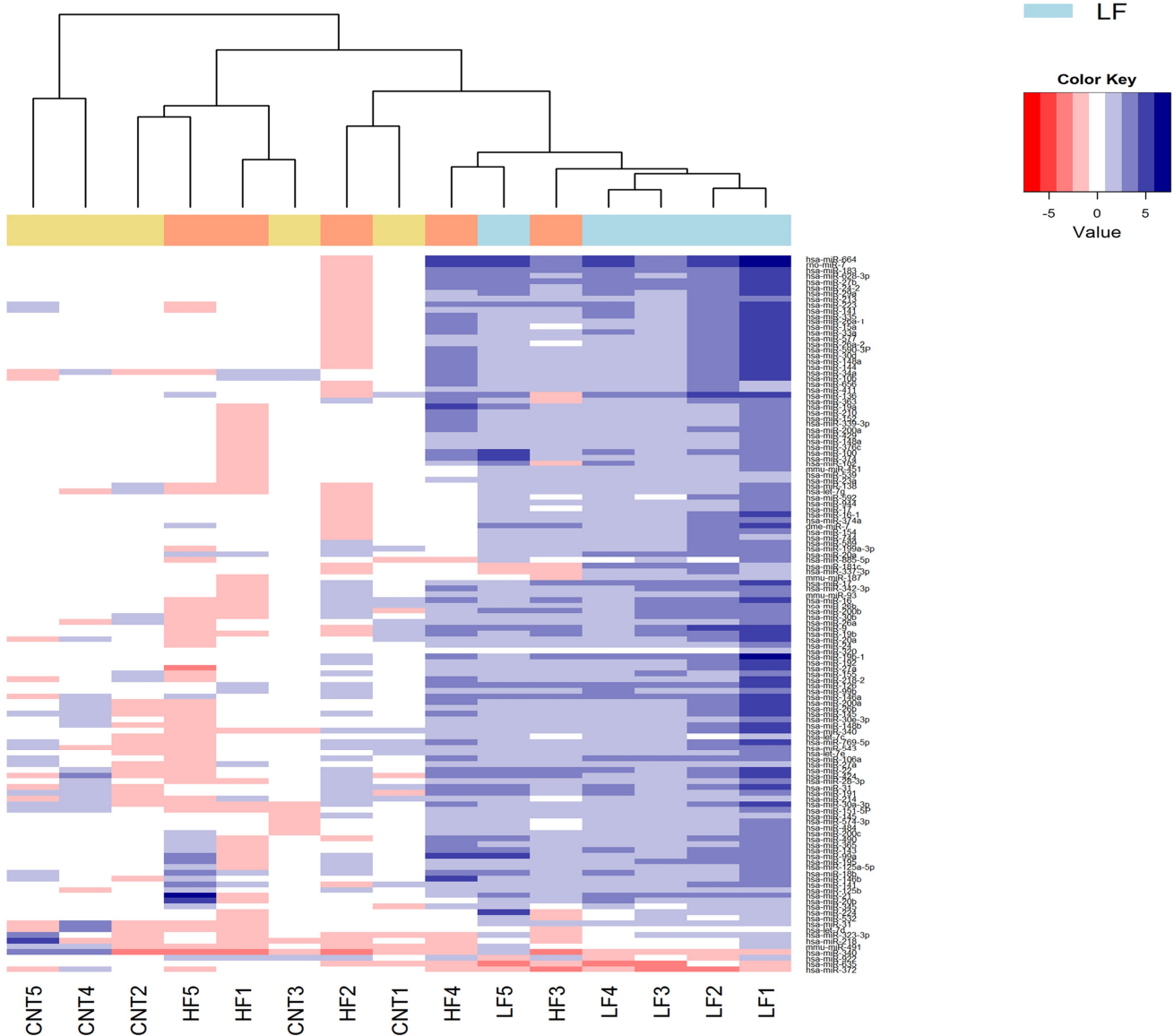
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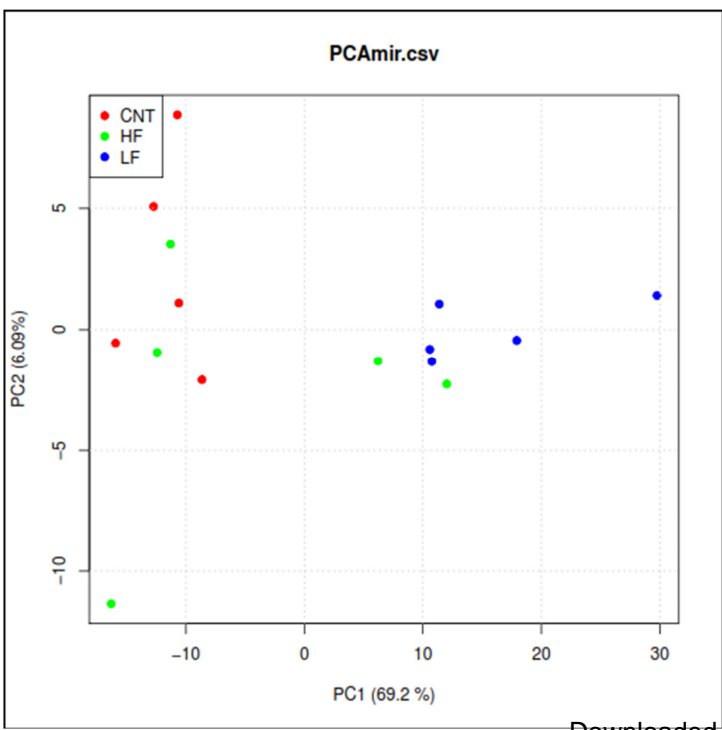
Fig. 1

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**log Relative Quantification
(Ward's clustering method)**



B



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Fig. 2

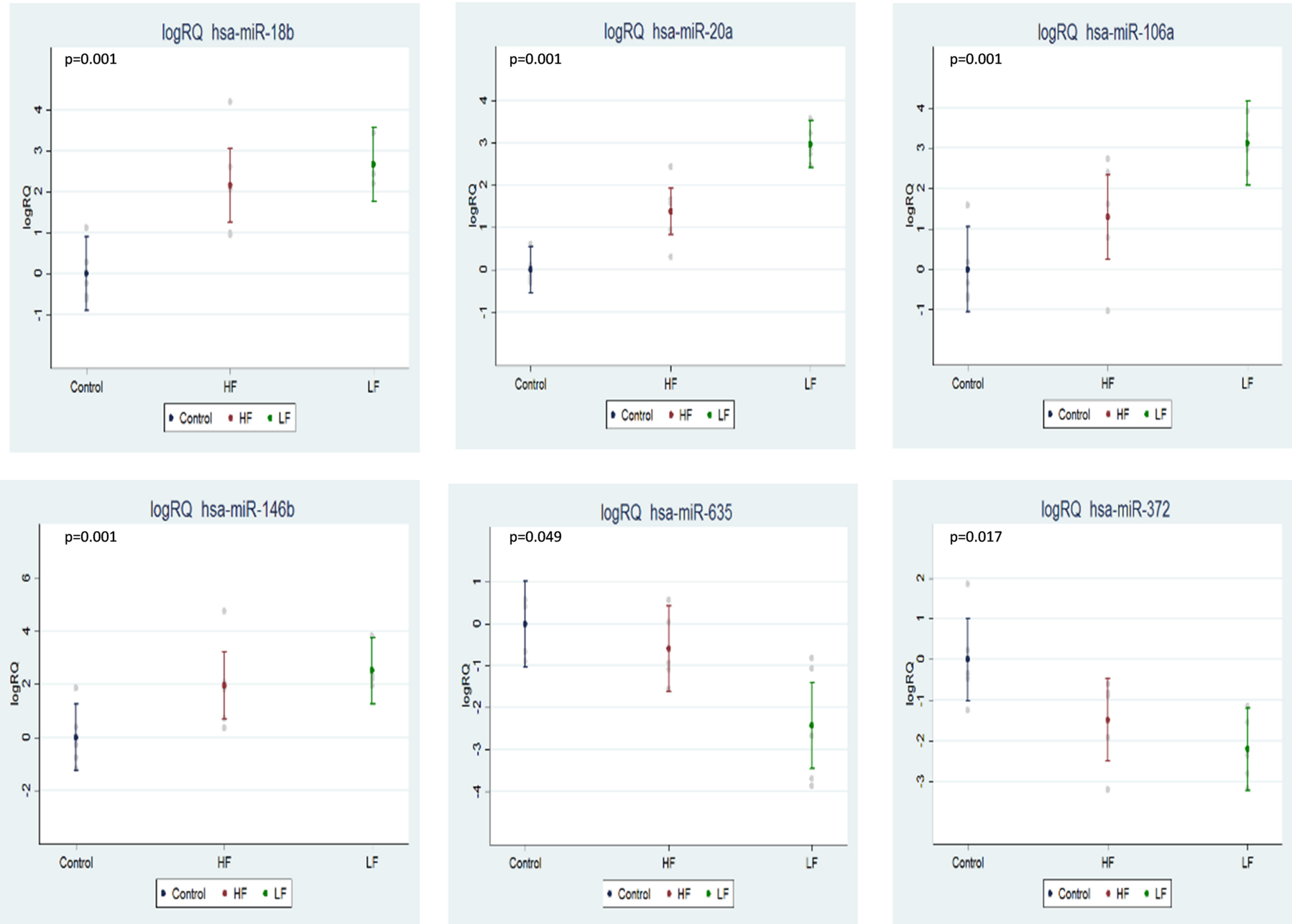
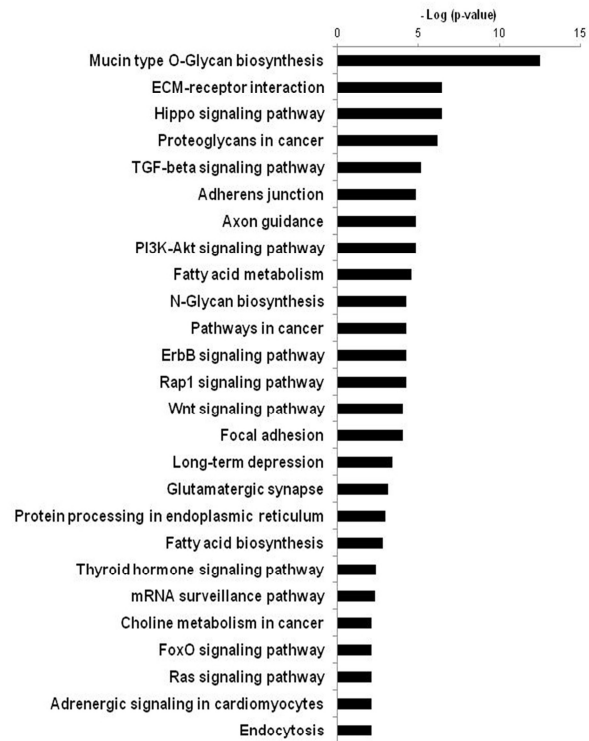


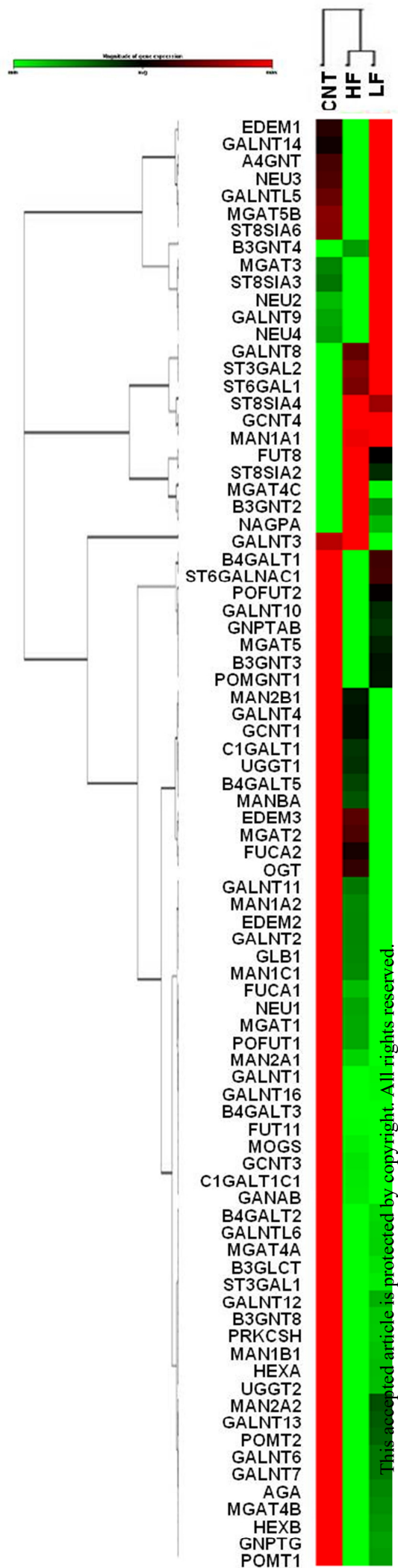
Fig. 3

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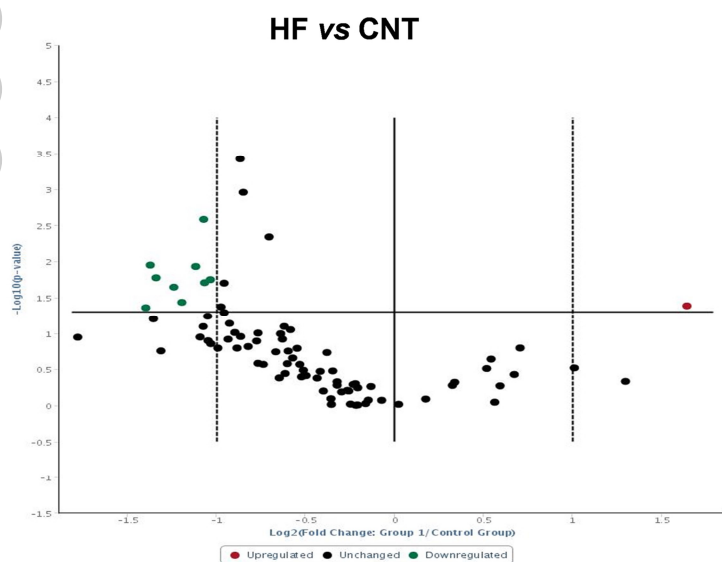
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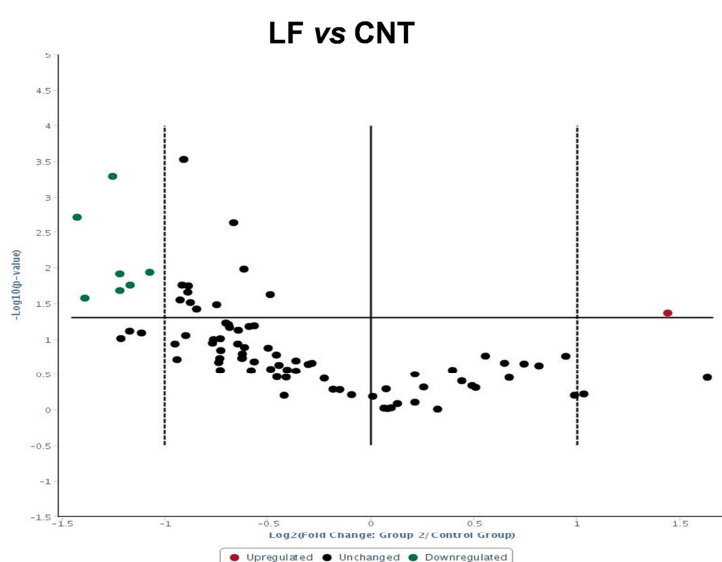
B



C



D



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Fig. 4

