Limited long-term impact of insect venom immunotherapy on the micro-RNA landscape in whole blood

Short title: Limited impact of insect immunotherapy on the miRNA in whole blood

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Abstract

Objective: To perform a genome-wide characterization of changes of microRNA (miRNA) expression in the course of VIT (venom immunotherapy).

Methods: miRNA was isolated form in the whole-blood of 13 allergic patients and 14 controls, who showed no allergic reaction upon stings by honeybees and wasps. We analyzed 2549 different miRNAs from whole blood of these patients prior to VIT and 12 months after the start of the VIT. Differential expression results obtained on microarray platform were confirmed by quantitative real -time PCR (qRT-PCR). Out of the 13 patients, eight were confirmed to show a negative allergic reaction upon VIT thus indicating a successful VIT.

Results: By comparing time points prior and 12 month after ultra-rush venom immunotherapy (VIT), correlation and principal component analysis both indicate a limited effect of the VIT on the overall miRNA expression pattern. Volcano blot analysis based on raw p-values revealed few deregulated miRNAs with the majority of them being increasingly expressed after VIT as compared to prior VIT. Using the 50 most altered miRNAs, there was no clear clustering according to the time points i.e. the time prior and the time after VIT.

Conclusions: Our results indicate an overall low effect of VIT on the miRNA expression pattern in whole blood.

Keywords

miRNA, Whole genome, Blood, Expression, Venom immunotherapy

Resumen

Objetivo: realizar la caracterización genómica de los cambios en la expresión de microARN (miARN) en el curso de ITV (inmunoterapia con veneno).

Métodos: Los microARNs se analizaron en la sangre total de 13 pacientes alérgicos y 14 controles sin reacción alérgica a las picaduras de abejas y avispas. Se analizaron 2549 miRNAs diferentes en la sangre total de estos pacientes antes de la ITV y 12 meses después del inicio de la ITV. Los resultados de expresión diferencial obtenidos en la plataforma de microarrays se confirmaron mediante PCR cuantitativa a tiempo real (qRT-PCR). De los 13 pacientes, se confirmó que ocho tenían una reacción alérgica negativa tras la ITV, lo que indicó una ITV exitosa. Resultados: al comparar los resultados de microRNAs, previa IT y 12 meses después de la (ITV), la correlación y el análisis de componentes principales indican un efecto limitado de la ITV en el patrón de expresión general de miARN. El análisis de Volcano basado en los valores de p crudos, reveló la existencia de pocos miRNAs desregulados estando la mayoría de ellos sobre-expresados tras la ITV en comparación con la previa. Utilizando los 50 miRNAs que más se alteraban, no se observó una agrupación clara en función del tiempo, es decir, pre y post-ITV.

Conclusiones: Nuestros resultados indican que la ITV tiene poco efecto en el patrón de expresión de miARN en sangre completa.

Palabras clave: miARN, genoma completo, sangre, expresión, inmunoterapia con veneno.

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Introduction

Stings by Hymenoptera, including honeybees and wasps, may cause systemic allergic reactions occurring with an estimated prevalence of 1–5 % in Europe [1]. Venom immunotherapy (VIT) is a frequently used procedure to effectively prevent further severe allergic reactions to insect stings in people who once had suffered such a complication [2-4]. While a number of immunologic alterations have been well characterized in the context of VIT, the underlying changes of gene expression and their relationship to the overall effectiveness of VIT still remain unclear [5]. Recently, microarrays have been used to identify venom allergy-associated modification of gene expression in peripheral blood (PB), even though PB is a heterogeneous mixture of different cell types [6-8]. A main goal of genome-wide gene expression research is to understand molecular triggers for venom allergy and identification of immunological pathways that are responsible for effective VIT response [9]. In the last decade, microRNAs (miRNAs) have emerged as key regulators in different human tissues and of diverse biological and pathological processes, including carcinogenesis, morphogenesis, apoptosis and cell differentiation [10-13]. MiRNAs are small single-stranded non-coding RNAs that anneal to the 3'-UTR of target mRNAs to down-regulate gene expression by inhibiting translation or stimulating mRNA degradation [14-17]. The involvement of miRNAs in various human diseases makes them promising novel targets of research providing insights into the molecular mechanisms of and possible therapeutically approaches in human malignancies [15]. Previous reports implicated miRNAs in the pathogenesis of several allergic diseases including asthma, food allergy, eosinophilic esophagitis, allergic rhinitis, and atopic dermatitis [18]. Most recently, several changes in miRNA expression have been identified in the build-up phase of hymenoptera VIT by screening 740 miRNAs [19]. In the present study, we studied the long-term effects of hymenoptera VIT by measuring the abundances of 2549 miRNAs prior and one year after the VIT.

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Materials and Methods

Study group. The group of patients selected for miRNA expression microarray consisted of 13 patients presenting severe allergic reactions (IIIo/IVo according to Mueller's classification) after bee or wasp sting (*Vespula* species) . Patients were recruited from the Department and Clinic of Internal Medicine and Allergology; Wroclaw Medical University, Poland, EU. As reference we included 14 individuals that showed no allergic reactions after bee or wasp stings. The study has been approved by a Wroclaw Medical University ethics committee (internal number 521/2012). All patients gave their informed consent to participate in the study. Blood from each patient has been sampled two times (prior and after one year of VIT). Detailed characteristics of individuals recruited to this study is presented in table 1.

Hymenoptera ultra-rush venom immunotherapy (VIT-UR) protocol: VIT-UR was performed in the same way for all study patients. VIT procedures were conducted according the ultra-rush protocol due to which treatment begins with the initial venom dose of 0.1 mcg and the summary dose of 111.1 mcg is reached within 3.5 hours. A venous access with infusion of sodium chloride 0.9% was secured before the first injection. Standardized lyophilized venom allergen extract (Pharmalgen Alk Abello) was used for build-up phase of VIT, and aluminum hydroxide adsorbed insect venom (Alutard-Alk Abello) for maintenance phase of therapy.

MiRNA isolation and microarray hybridization: Whole blood samples were collected in PAXgene Blood RNA Tubes (BD, Franklin Lakes, New Jersey, USA) and stored in -60°C. Total RNA containing miRNA was isolated using PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany). MiRNA abundances were analyzed using miRNA expression array (SurePrint G3 Unrestricted miRNA 8x60K v21, Agilent Technologies, Santa Clara, CA, USA) encompassing 2549 known human miRNAs. All procedures were carried out according to the manufacturer's recommendations: To generate fluorescently labeled RNAs, 100ng total RNA per sample was processed by the miRNA Complete Labeling and Hyb Kit (Agilent Technologies, Santa Clara, CA, USA). Samples were loaded onto the microarrays and incubated for 55°C for 20 hours with rotation. Subsequent to washing microarrays were scanned with the Agilent Microarray Scanner at 3 microns in double

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path mode. Data were acquired using Agilent AGW Feature Extraction software version 10.10.11 (Agilent Technologies, Santa Clara, CA, USA).

Real-time quantitative reverse transcription-PCR (RT-qPCR) analysis: Expression levels of 2 miRNAs that displayed significant deregulation in T cells CD8 were analyzed on the prepared cDNA using the miScript II RT Kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Detection of amplification was performed on 5 ng of cDNA on an LightCycler480 instrument (Roche, USA) using the miScript Starter PCR Kit (Qiagen) and miRNA specific quantitative RT-PCR primer sets for the miRNA of interest (Qiagen): hsa-miR-16-2 (cat.no: MS00008813) and hsa-miR-4516 (cat.no: MS00037555) and endogenous control RNU6-2 (included in the miScript Starter PCR Kit). Each qRT-PCR reaction was conducted in duplicate.

Statistical analysis: We used the freely available R statistical environment v.3.4.0 to analyze the differences of miRNA abundance in VIT patients. After applying the Feature Extraction software, we computed the normalized total gene signal for each miRNA by performing the RMA method (AgiMicroRna) based on quantile normalization [20]. The resulting expression values were transformed to log (base 2). Then the whole miRNA set of 2,549 miRNAs was reduced to 534 by filtering in that way that each marker was detected at least in 25% of the samples in one of the 3 groups (before, after and reference). Different levels of miRNAs among the same probands before and after the wasp sting were analyzed with using a paired two-tailed t test. We carried out a hierarchical clustering approach on top 50 most altered miRNAs (as revealed by raw p-values) to detect clusters of miRNAs and blood samples. In detail, we applied bottom up complete linkage clustering and used the Euclidian distance measure. In addition, we carried out a standard principal component analysis (PCA) and provide scatter plots of the first versus second principal component [21]. Relative quantification of miRNA expression and calculation of the range of confidence was performed using the REST 2009 Software [22].

Computation blood cell-specific differential expression: For computation of relative blood cell proportions, we used normalized gene expression data obtained from the same patients on the Affymetrix PrimeView platform (results to be published, raw data available from Geo Expression Omnibus: GSE92866). After data normalization, we

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utilized CIBERSORT algorithm (1000 iterations) and LM22 gene signature to predict relative proportions 9 major cell types (B cells, T cells CD8, T cells CD4, T cells regulatory (Tregs), NK cells, monocytes, macrophages, dendritic cells and neutrophils) [23]. Calculated proportions were used as an input to csSAM package that allows the analysis of differential expression for each blood cell type [24].

Results

The aim of the study was to provide a comprehensive comparison of miRNAs derived from whole blood of patients prior and 12 months after the start of VIT treatment (referred to as „after VIT"). In total, we compared the abundance of 2549 miRNAs from 13 patients including 6 males and 7 females, each showing severe allergic reactions of grades IIIo or IVo according to Mueller's classification after bee or wasp stings. Detailed clinical characteristics of the patients are given in table 1. Out of the 13 patients, eight were challenged with insect venom after finishing therapy and all eight patients were negative for signs of allergy against Hymenoptera venom, indicating a successful VIT. The control group (7 males and 7 females) consisted of individuals without allergic reactions after bee or wasp sting (as revealed by skin prick test).

We first compared the individuals tested based on the overall miRNA expression pattern. The correlation analysis between the samples identified several samples with a reduced correlation (Figure 1). Principal component analysis (PCA) showed no signs of difference between the time points prior and 12 months after VIT (Figure 2). As for specific miRNAs with abundances altered by VIT, volcano blot analysis based on raw p-values revealed few deregulated miRNAs with the majority of them displaying a lower abundance level prior to VIT as compared to after VIT (Figure 3a). After p-value adjustment, the volcano blot analysis did not indicate any miRNA with a significantly altered abundance between the time point prior and after VIT (Figure 3b). These results also point to an overall low effect of VIT on the miRNA expression pattern in whole blood. Figure 4 presents exemplary results of ANOVA analysis between both time points and the controls.

Based on the differences in miRNA abundance between the time points prior and after VIT we performed a clustering analysis using the 50 most altered miRNAs. Figure 4

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shows the lack of clustering between two time points prior and after VIT. These data further indicate a rather limited impact of VIT-UR on miRNA expression pattern in blood.

Finally we computed differential expression for each blood cell type between two time points (prior and after VIT). Applying this approach we found 14 miRNAs (4 downregulated / 10 upregulated) with a differential abundance in T CD8 lymphocytes and 2 differentially expressed miRNAs in monocytes (adjusted p-value ≤0.25 as proposed by authors of csSAM; see ref. (Shen-Orr, et al., 2010)) (Table 2). Subsequently, we selected two miRNAs based on significant deregulation in T CD8 cells (hsa-miR-16-2-3p and hsa-miR-4516) to preform qRT-PCR in the whole blood (Figure 5). None of selected miRNAs revealed significant alteration when comparing our study groups (prior and after VIT).

Functional enrichment of genes targeted by up- or downregulated miRNAs revealed a number of significantly deregulated pathways, however, none of them could be attributed to immunology- or allergy-related mechanisms (data not shown).

Discussion

Most recently, several changes in miRNA expression have been identified in the build-up phase of hymenoptera VIT by screening 740 miRNAs [19]. Here, we studied the long-term effects of hymenoptera VIT by measuring the abundances of 2549 miRNAs one year after the VIT. While we found a limited effect on the overall miRNA expression pattern, we nevertheless detected few miRNAs that displayed an altered expression pattern one year after VIT. The influence of the VIT was sufficient to prevent clustering of the two samples that belong to a given patient.

The difference in results of our and the study of Specijalski et al. (2016) can be explained the time points taken for the buildup phase of the immunotherapy: measuring of miRNA changes 24 hours after VIT vs. 12 months, as in our study [19]. Further differences between both studies include the mode of blood drawing (PAXgene versus conventional blood collection), the numbers of miRNAs analyzed (2549 miRNAs vs 740 miRNAs) and different methods used (array based approach vs qRT-PCR). While bearing these facts in mind the comparison of both studies indicates that the long and short term effects of VIT on the miRNA pattern appear to be different. After 12 month upon VIT, the whole-blood

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miRNA expression pattern was largely similar to the pattern prior to venom immunotherapy.

As in numerous previous studies we used the PAXGene system that allows immediate stabilization of intracellular RNA, thereby facilitating the generation of reproducible miRNA expression data. A drawback of this approach is that the lysis of blood prevents to relate the identified miRNA pattern to specific blood cells. In addition, even experimental cell sorting does not necessarily allow to detect the miRNA pattern of specific cell type due to the stress effects on expression patterns as previously described by us and others [25,26]. To address this problem, we tested a deconvolution algorithm that allows to predict differently expressed miRNAs in small cell subpopulations [27]. Using the deconvolution algorithm, we predicted the miRNA abundance in B cells, T cells CD8, T cells CD4, T cells regulatory (Tregs), NK cells, monocytes, macrophages, dendritic cells and neutrophils. We found 14 miRNAs with a differential abundance in TCD8 lymphocytes and two miRNAs in monocytes. The elevated number of miRNAs predicted to be deregulated in T cells is consistent with previous studies suggesting that biochemical changes in CD8+ T-cell function may represent key events in successful T-cell immunotherapy [28]. As for the function of monocytes in course of VIT, there is less evidence linking changes of miRNA abundance to VIT. Although the employed deconvolution algorithm indicates differently expressed miRNAs in blood cell subpopulations, the influence on cell type specific miRNA changes by VIT awaits experimental validation.

Potential limitation of our study is the fact that that whole blood was used as the miRNA source, whereas, significant expression changes in VIT revealed by deconvolution approach were specific to a small fraction of cells (e.g T cells CD8). Presence of large proportion of cells not determining subject condition in the context of VIT (e.g. neutrophils) may weaken cell-specific expression signals.

In summary, we studied the influence of hymenoptera VIT on the miRNA abundance in whole blood of Hymenoptera venom allergic patients and found evidence for expression changes of specific miRNA in whole blood between patients prior and 1 year after VIT. The overall miRNA pattern was, however, largely unaffected. Our results lay the ground to further study the role of a miRNA-based regulation during the process of desensitization, including cell-type specific alteration of miRNA abundances.

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Conflicts of interest

None to declare

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Table 1. Demographic and clinical data

Patient's Number (A-prior, C-after, G - controls)	Age	Gender	Grade of allergic reaction after sting According to Mueller scale	Responsible insect	IDT* with venom of responsible insect - concentration 10^{-6}g/l (mm)	sIgE** with venom of responsible insect (concentration and class)	Vesp v 1 (concentration and class)	Vesp v 5 (concentration and class)	Api m 1 (concentration and class)	Results of sting challenge / field sting
11A/11C	45	F	III	Wasp	12x12	12.13 (2)	0.02 (0)	6.82 (3)	0.002 (0)	Negative
6A/6C	32	M	IV	Wasp	9x11	2.23 (2)	0.03 (0)	10.4 (3)	0.01 (0)	Negative
2A/2C	45	M	IV	Wasp	10x10	0.589 (1)	0.07 (1)	0.33 (0)	0 (0)	Negative
7A/7C	40	M	IV	Wasp	11x14	9.15 (3)	11.4 (3)	0.28 (0)	0 (0)	Negative
19A/19C	37	M	IV	Wasp	8x11	1.86 (2)	ND***	ND	ND	Negative
15A/15C	40	F	III	Wasp	10x12	7.59 (3)	0.61 (1)	3.47 (2)	0 (0)	Negative
13A/13C	50	F	IV	Wasp	10x11	5.11 (3)	1.96 (2)	4.88 (3)	0.37 (1)	ND
16A/16C	62	M	III	Bee	13x16	8.96 (3)	ND	ND	ND	Negative
1A/1C	38	F	IV	Wasp	7x9	5.2 (3)	0.01 (0)	3.12 (2)	0.004 (0)	ND
14A/14C	62	F	IV	Wasp	8x10	0.35 (0)	0.003 (0)	1.59 (2)	0 (0)	ND
18A/18C	56	F	IV	Wasp	7x8	2.15 (2)	ND	ND	ND	Negative
17A/17C	71	M	III	Bee	10x12	5.61 (3)	0 (0)	0 (0)	0.12 (0)	ND
13A/13C	52	F	III	Wasp	10x11	1.11 (3)	10.96 (2)	1.88 (3)	0.67 (0)	ND
10G	23	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
11G	22	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
12G	25	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
13G	31	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND
14G	22	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
1G	33	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
2G	27	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
3G	29	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND
4G	26	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND
5G	NA	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND
6G	60	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND
7G	18	M	Non-allergic	ND	ND	ND	ND	ND	ND	ND
8G	62	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND
9G	28	F	Non-allergic	ND	ND	ND	ND	ND	ND	ND

*IDT - intradermal test; ** sIgE – specific IgE; ***ND - not determined

Table 2. Differentially regulated miRNAs in blood cell types

T CD8	Status	Expression difference*	FDR** (p-value)
hsa-miR-16-2-3p	downregulated	-3.66	0.21
hsa-miR-371b-5p	upregulated	3.19	0.21
hsa-miR-3960	upregulated	4.44	0.21
hsa-miR-4286	downregulated	-3.41	0.21
hsa-miR-4466	upregulated	3.00	0.24
hsa-miR-4516	upregulated	3.91	0.21
hsa-miR-4763-3p	upregulated	3.32	0.21
hsa-miR-6089	upregulated	3.54	0.21
hsa-miR-638	upregulated	3.17	0.21
hsa-miR-664a-3p	downregulated	-3.21	0.21
hsa-miR-6803-5p	upregulated	3.26	0.21
hsa-miR-6826-5p	downregulated	-3.08	0.23
hsa-miR-7108-5p	upregulated	3.92	0.21
hsa-miR-8063	upregulated	3.27	0.21
Monocytes			
hsa-miR-6785-5p	upregulated	4.89	0.23
hsa-miR-494-3p	upregulated	4.39	0.23

* Expression difference=after - prior
**FDR cutt off<=0.25

Figure Legends

Figure 1. Correlation matrix. Pair-wise Pearson Correlation Coefficient of the miRNA expression profile was computed for all samples. Yellow to blue colors indicate high or lower correlation coefficient values. The numbers indicate the different individuals, A and B denotes the time point of blood collection prior and after VIT, respectively. Controls are indicated by G.

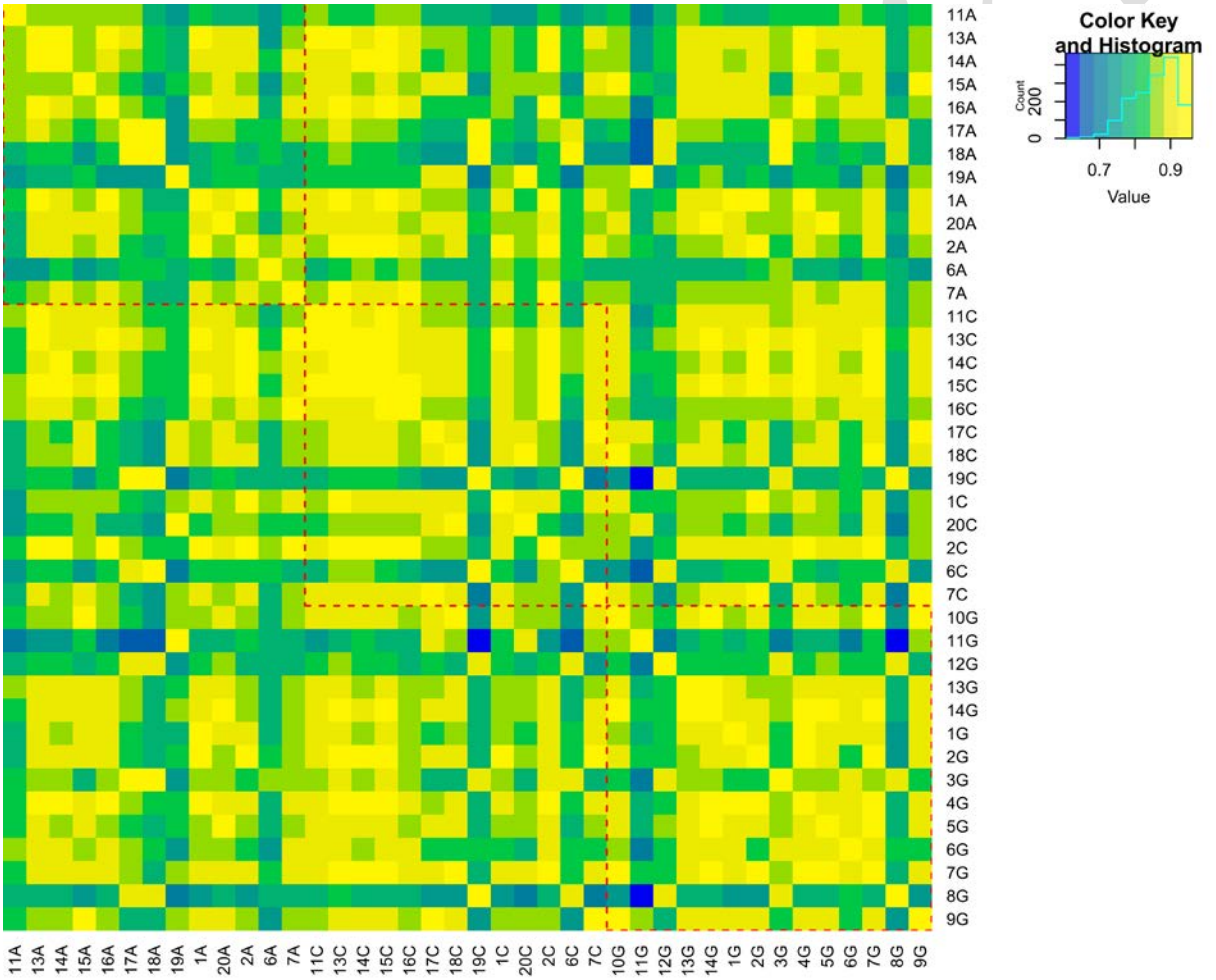

Figure 2. Principal Component Analysis. Principal Component Analysis (PCA) of blood samples taken from patients before VIT and 12 months after VIT. The figure shows the first principal component (PC1) on the x-axis versus the second PC2 on the y-axis. The numbers indicate the different patients, A and B denotes the time point of blood collection prior (green) and after VIT (orange), respectively. Controls are indicated in blue and by G.

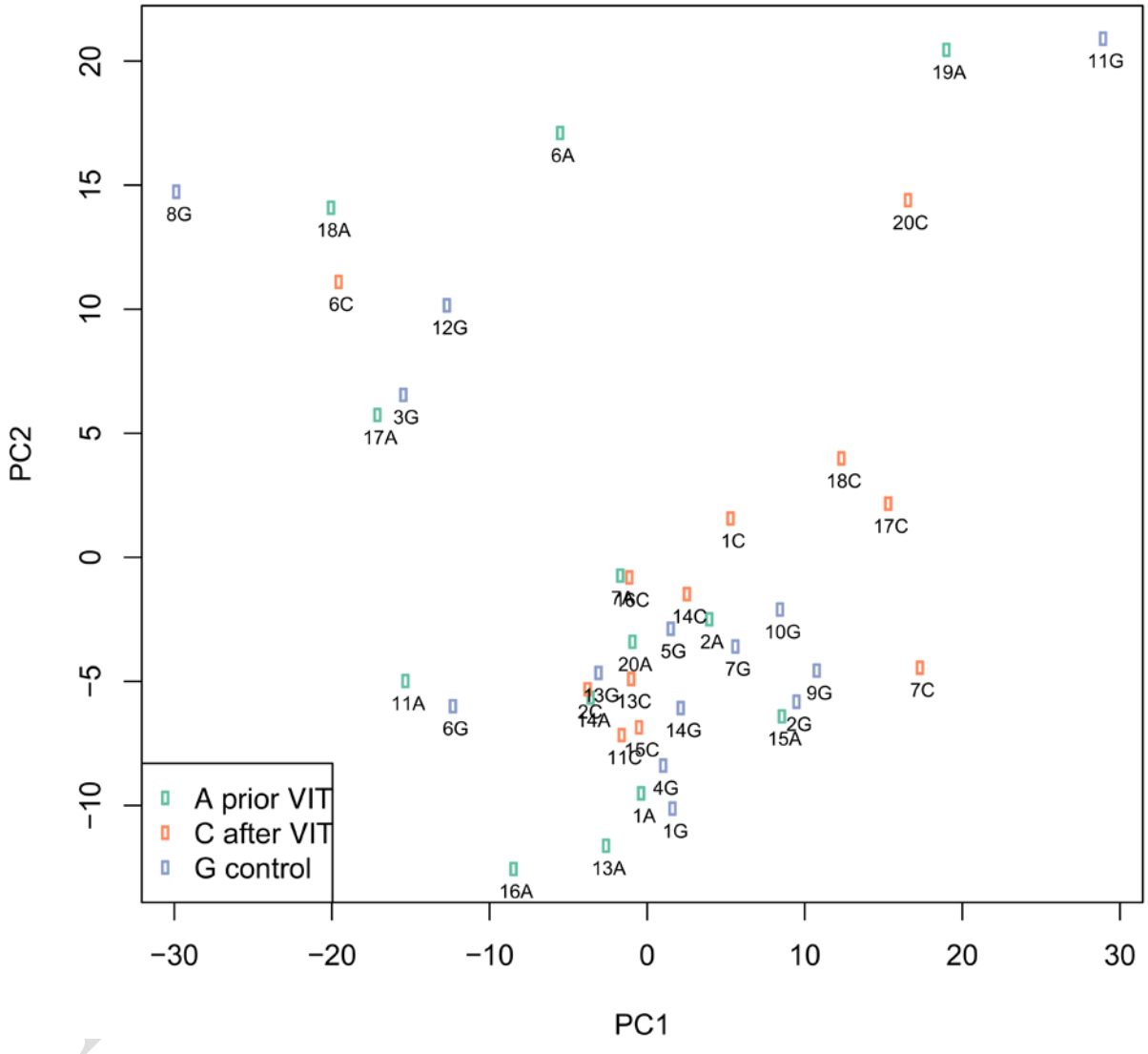

Figure 3. Differential expression of miRNAs upon VIT. A - shows analysis based on raw values; **B** - shows data after adjustment of p-value for multiple testing. Volcano plots of all measured blood borne miRNAs show the fold change of each miRNA in logarythmic scale (base 2) on the x-axis, and the negative logarythmic p-value (base 10) on the y-axis. The miRNA significantly overexpressed prior to IVT versus after IVT are indicated as red dots. MiRNAs with a significantly lower expression prior IVT are indicated as green dots.

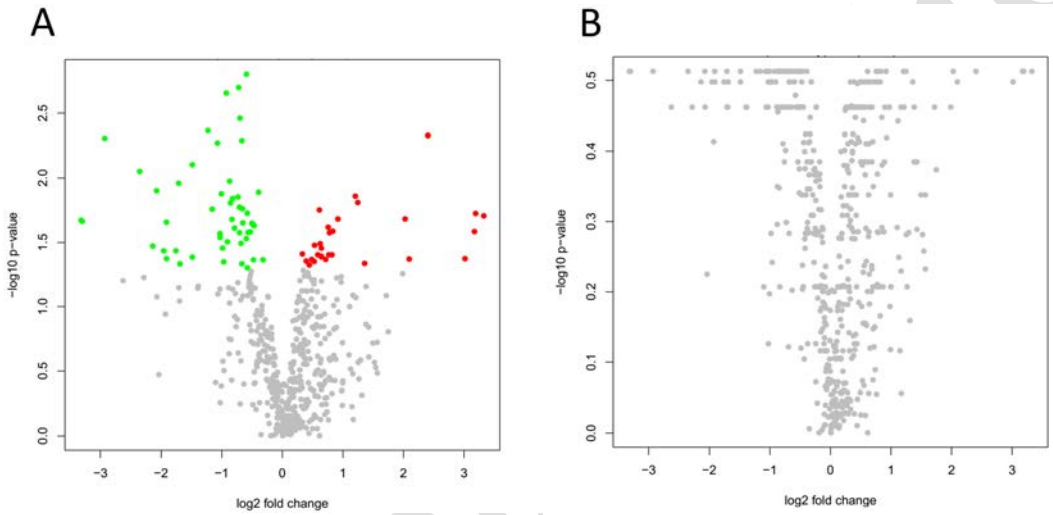

Figure 4. Hierarchical clustering of samples before and after VIT based on top most altered 50 miRNAs. The figure presents hierarchical clustering in patients prior VIT (green) and 12 months after the start of the VIT (orange). Controls are indicated in blue. Samples belonging to the same individual are indicated at the bottom of the heat map; miRNAs used for the heat map are indicated at the right-hand side.

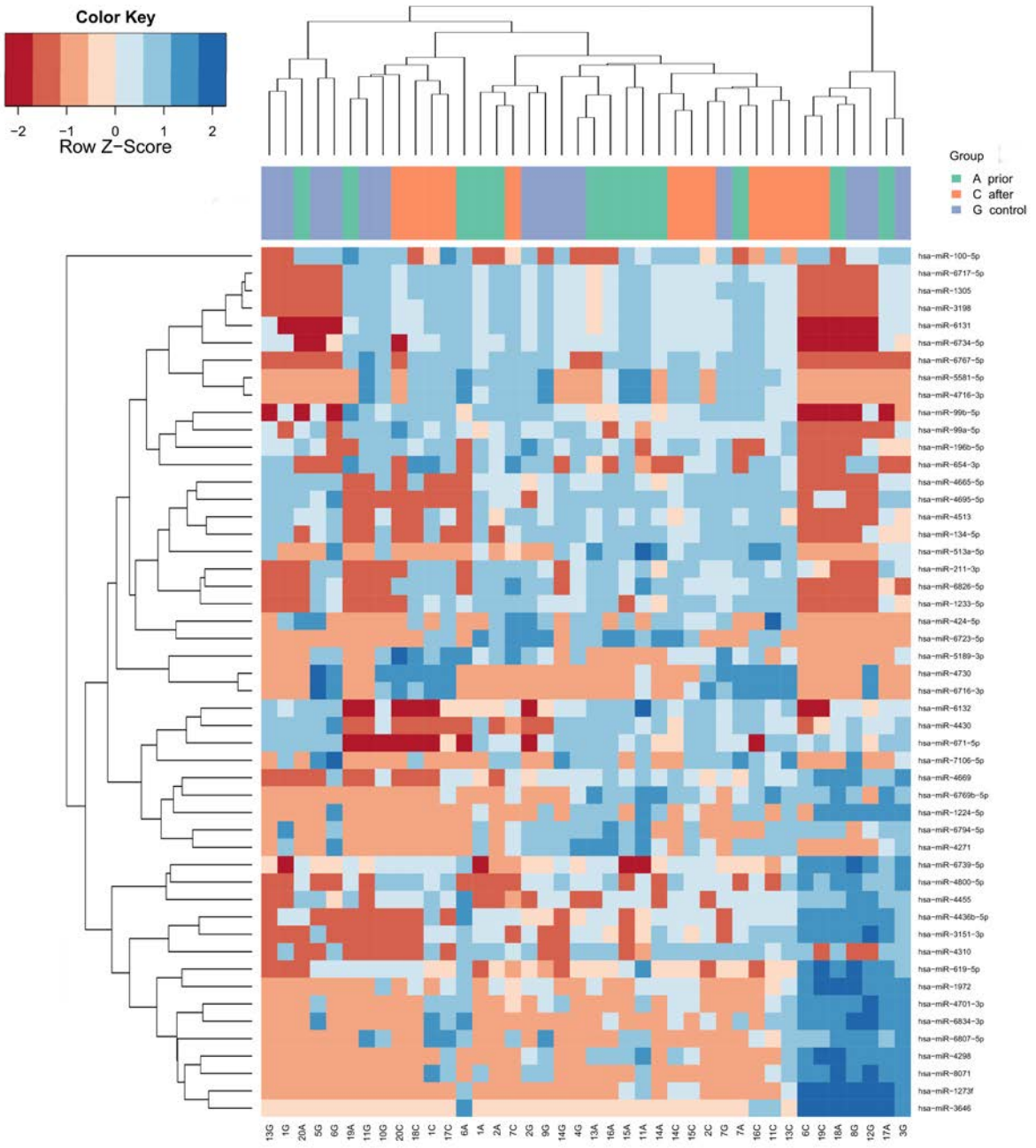

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Figure 5. Expression of 2 selected miRNA before and after VIT. A - derived from array based approach. **B** - derived from RT-qPCR. Box-whisker plots of miRNAs differentially expressed in blood of patients prior IVT is indicated in red and 12 months after VIT indicated in green The boxes represent the 2nd and 3rd quartile, with the whiskers displaying minimum and maximum expression values. MiRNAs were selected based on significant deregulation in T cells CD8 (see table 2).