NEXT-GENERATION SEQUENCING (NGS) OF TWO INDEPENDENT COHORTS IDENTIFIES ELEVEN CIRCULATING miRNAs FOR DIAGNOSIS OF NASH AND FIBROSIS

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BACKGROUND

The prevalence of NASH is rapidly growing and represents a major public health issue. NASH drives progressive fibrosis accumulation and ultimately leads to cirrhosis and hepatocellular carcinoma (HCC). Although there is no current pharmacological treatment for NASH, many new drug candidates are currently in phase 2 and phase 3 and new treatments should be available in the coming years.

Liver biopsy remains the only method for diagnosis of NASH and for scoring disease activity and stage of liver fibrosis. However, biopsy is invasive and can not be used in the large number of patients that may be in need for pharmacotherapy. There is an urgent need for new and widely available non-invasive diagnostic methods for NASH.

Micro-RNAs (miRNAs) can play an important role in regulating gene expression in chronic liver diseases including NASH. Importantly, miRNAs are released into the extracellular space and body fluids, where they remain remarkably stable. Circulating miRNAs have been investigated in a wide variety of NAFLD/NASH animal models, as well as in small cohorts of patients and represent innovative candidates for the development of new *in vitro* diagnostic tests.

AIMS

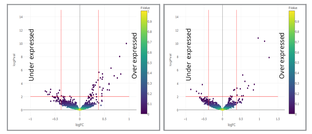
- Several reports have explored miRNAs in circulation of control, simple steatosis and NASH patients, providing evidence for using miRNAs as potential diagnostic or prognostic biomarkers for NASH (see Alfonso MB *et al.*, J. Clin Med. 2016;5(3):30). These studies mainly measured plasma/serum levels of a limited number of miRNAs, which were selected based on previous reports of their association with liver diseases, cancer, or CVD.
- Our aim was to perform an unbiased identification of miRNAs differentially expressed in serum samples from patients with NASH and fibrosis (To-Be-Treated, TBT) vs serum samples from patients without indication for pharmacotherapy because of low disease activity and/or low fibrosis stage (Not-To-Be-Treated). We used two large independent cohorts of patients with scored liver biopsy and corresponding serum samples. Circulating levels of 2083 miRNA species were simultaneously measured and discriminating miRNAs were then confirmed by a classical RT-qPCR approach.

METHODS

- A total of 517 serum samples from two independent patient cohorts and corresponding liver biopsies were used (N=269 for GOLDEN-Diag cohort ; N=248 for OBESE cohort).
- Serum levels of 2083 miRNAs (miRBase) were measured using HTG-EdgeSeq-NGS technology.
- The levels of miRNAs (number of reads) in serum samples of NASH patients at risk of fibrosis progression (To-Be-Treated; TBT=NAS≥4, F≥2 at histological exam) were compared to levels obtained in serum of Not-To-Be-Treated (NTBT) patients. Fold change (TBT vs NTBT) and statistical significance were calculated by dedicated bioinformatic approaches.
- Differentially expressed miRNAs were sorted by fold change and/or statistical significance (p<0.01) in the two cohorts.
- Most relevant miRNAs were then measured by RT-qPCR in all serum samples in the two cohorts.
- Correlations of circulating levels of each relevant miRNAs with NAFLD-Activity score (NAS), Activity Index (hepatocyte ballooning + lobular inflammation) and fibrosis stage were assessed.
- ROC analyses were performed for each relevant miRNA.

RESULTS: HTG-EdgeSeq-NGS

Volcano-plots obtained in GOLDEN-Diag and OBESE comparing fold change and p value of each individual miRNA in TBT vs NTBT patients



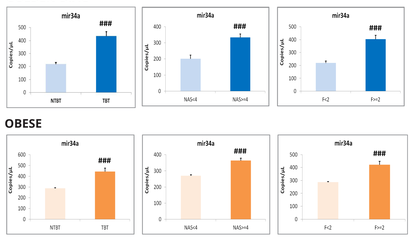
Commonly over-expressed miRNAs (TBT vs NTBT; p<0.01 and fold change >1.3) in GOLDEN-Diag and OBESE

	GOLDEN (N=269) 109 TBT vs 160 NTBT		OBESE (N=248) 50 TBT vs 198 NTBT	
	Fold Change	p-value	Fold Change	p-value
mir34-5p	**1.92**	**1.3E-10**	**2.18**	**5.5E-11**
mirA	1.76	8.1E-09	1.96	1.2E-11
mirB	1.55	4.9E-07	1.81	1.7E-04
mirC	1.38	4.2E-06	1.45	7.0E-06
mirD	1.37	1.8E-05	1.52	5.6E-05
mirE	1.33	2.4E-05	1.31	6.6E-03
mirF	1.37	2.9E-05	1.40	7.2E-04
mir122-5p	**1.50**	**2.0E-04**	**2.40**	**1.5E-08**
mirG	1.38	8.6E-04	1.34	4.0E-02
mirH	1.37	1.2E-03	1.62	2.7E-04
mirI	1.44	1.7E-03	1.48	7.5E-03

- Volcano plot derived from comparison of serum levels of 2083 different miRNAs in TBT and NTBT are presented in Figure 1.
- There were more over-expressed than under-expressed miRNAs when selection was based on p<0.01:
  - 34 over-expressed vs 14 under-expressed in GOLDEN-Diag
  - 17 over-expressed vs 5 in OBESE
- When selection of over-expressed miRNAs was based both on statistical significance (p<0.01) and fold change (>1.3) in TBT vs NTBT:
  - 21 were selected in GOLDEN-Diag
  - 14 were selected in OBESE
- After removing miRNAs with low expression levels in GOLDEN-Diag (<100 reads in both TBT and NTBT), cross-validation between the two cohorts gave the following list of commonly over-expressed miRNA in TBT vs NTBT (see Table).
- As expected, mir34a and mir122 were selected among discriminating miRNAs.

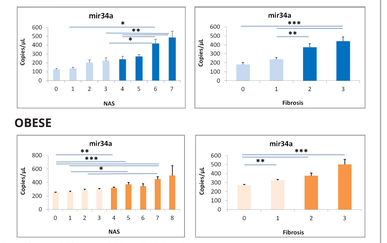
CONFIRMATION OF mir34a AND mir122 BY RT-qPCR

Discriminating potency of mir34a in GOLDEN-Diag and OBESE on: TBT vs NTBT, NAS≥4 vs NAS<4 and F≥2 vs F<2



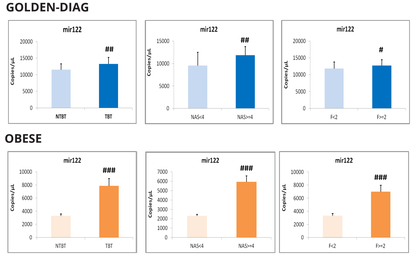
Mean±SE, ###p<0.001, Mann-Withney test

Correlation of mir34a serum level with NAS and Fibrosis score



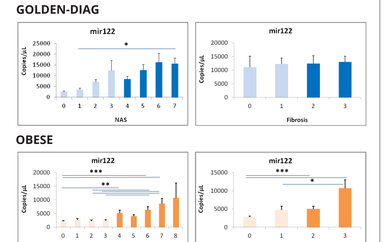
*Mean±SE, ***p<0.001, **p<0.01, *p<0.05, Dunn's test*

Discriminating potency of mir122 in GOLDEN-Diag and OBESE on: TBT vs NTBT, NAS≥4 vs NAS<4 and F≥2 vs F<2



Mean±SE, ###p<0.001, ##p<0.01, #p<0.05, Mann-Withney test

Correlation of mir122 serum level with NAS and Fibrosis score

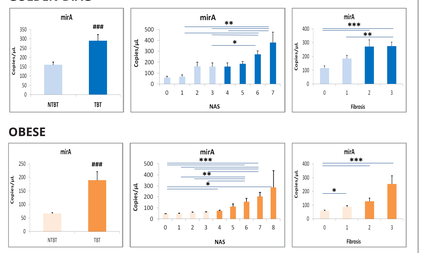


*Mean±SE, ***p<0.001, **p<0.01, *p<0.05, Dunn's test*

- RT-qPCR experiments confirm a strong differential expression of both mir34a and mir122 in the two cohorts.
- In both cohorts, mir34a serum levels gradually increase with NAS and fibrosis.
- In contrast mir122 levels increased with NAS in both cohorts but not with fibrosis in the GOLDEN-Diag cohort.

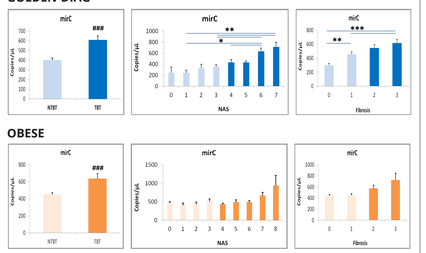
CONFIRMATION OF NEWLY IDENTIFIED miRNAs BY RT-qPCR

Discriminating potency of mirA and correlation with NAS and Fibrosis score



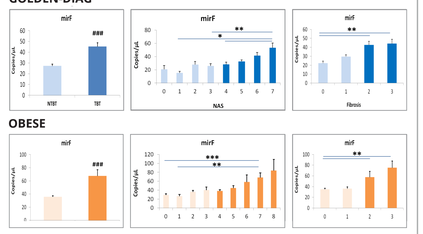
*Mean±SE, ###p<0.001, Mann-Withney test; ***p<0.001, **<p<0.01, *p<0.05, Dunn's test*

Discriminating potency of mirC and correlation with NAS and Fibrosis score



*Mean±SE, ###p<0.001, Mann-Withney test ***p<0.001, **<p<0.01, *p<0.05, Dunn's test*

Discriminating potency of mirF and correlation with NAS and Fibrosis score



*Mean±SE, ###p<0.001, Mann-Withney test; ***p<0.001, **<p<0.01, *p<0.05, Dunn test*

Fold change and AUROC of individual miRNAs dosed by RT-qPCR

	GOLDEN-Diag (N=269) 109 TBT vs 160 NTBT			OBESE (N=248) 50 TBT vs 198 NTBT		
	Fold Change	p-value	AUROC	Fold Change	p-value	AUROC
mir34a-5p	**1.99**	**<0.001**	**0.74**	**1.54**	**<0.001**	**0.74**
mirA	1.80	<0.001	0.68	2.85	<0.001	0.74
mirC	1.52	<0.001	0.68	1.41	<0.001	0.65
mirF	1.65	<0.001	0.65	1.88	<0.001	0.68
mir122-5p	**1.15**	**<0.01**	0.59	**2.39**	**<0.001**	**0.77**
mirG	1.65	<0.01	0.61	1.18	<0.01	0.62
mirH	1.25	<0.01	0.63	1.12	<0.01	0.62

- RT-qPCR experiments confirm that mirA, mirC and mirF were discriminating miRNAs in both cohorts.
- mirA and mirF had high discriminating potencies in both cohorts and their serum levels significantly increased with NAS and fibrosis stage.
- mirC had a high discriminating potency in both cohorts but there was no significant correlation with NAS and fibrosis stage in OBESE.

CONCLUSIONS

- **This analysis of more than 500 NAFLD patients from two independent cohorts by NGS technology, allowed a non-biased selection of the most discriminating circulating miRNAs associated with NASH and liver fibrosis.**
- **From a total of 2083 miRNAs reffered in miRBase, 11 miRNAs were significantly over expressed in TBT vs NTBT in two independent cohorts of patients.**
- **In addition to mir34a and mir122, 9 new miRNAs were significantly associated with To-Be-Treated condition, NAS≥4 and F≥2.**
- **RT-qPCR experiments confirmed that serum levels of selected miRNAs gradually increased with NAS and fibrosis score.**
- **These miRNAs hold promise for developing new *in vitro* diagnostic tests to non-invasively screen NAFLD patients and identify NASH patients To-Be-Treated.**

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