Blood hsa-miR-122-5p and hsa-miR-885-5p levels associate with fatty liver and related lipoprotein metabolism—The Young Finns Study

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MicroRNAs are involved in disease development and may be utilized as biomarkers. We investigated the association of blood miRNA levels and a) fatty liver (FL), b) lipoprotein and lipid pathways involved in liver lipid accumulation and c) levels of predicted mRNA targets in general population based cohort. Blood microRNA profiling (TaqMan OpenArray), genome-wide gene expression arrays and nuclear magnetic resonance metabolomics were performed for Young Finns Study participants aged 34–49 years (n = 871). Liver fat status was assessed ultrasonographically. Levels of hsa-miR-122-5p and -885-5p were up-regulated in individuals with FL (fold change (FC) = 1.55, p = 1.36 × 10−14 and FC = 1.25, p = 4.86 × 10−4, respectively). In regression model adjusted with age, sex and BMI, hsa-miR-122-5p and -885-5p predicted FL (OR = 2.07, p = 1.29 × 10−8 and OR = 1.41, p = 0.002, respectively). Together hsa-miR-122-5p and -885-5p slightly improved the detection of FL beyond established risk factors. These miRNAs may be associated with FL formation through the regulation of lipoprotein metabolism as hsa-miR-122-5p levels associated with small VLDL, IDL, and large LDL lipoprotein subclass components, while hsa-miR-885-5p levels associated inversely with XL HDL cholesterol levels. Hsa-miR-885-5p levels correlated inversely with oxysterol-binding protein 2 (OSBPL2) expression (r = −0.143, p = 1.00 × 10−4) and suppressing the expression of this lipid receptor and sterol transporter could link hsa-miR-885-5p with HDL cholesterol levels.

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Fatty liver (FL) is characterized by the accumulation of triacylglycerol-rich lipid droplets in hepatocytes (liver fat > 5–10% of liver weight). The primary causes leading to hepatocellular lipid accumulation are not yet well understood, but they are thought to include alterations in the hepatic lipid uptake, synthesis, degradation, and secretion1. FL is the first stage of a continuum from benign, simple steatosis to steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma2. Non-alcoholic FL disease (NAFLD) is defined as FL without evidence of excessive alcohol intake or other causes of steatosis and it has histological and pathological similarities with alcoholic liver disease (ALD)3. NAFLD is common in Western and Asian populations with a prevalence of 20–30% in the general population and 70–90% among the obese and diabetics4. It is also frequently associated with obesity, type 2 diabetes, and hyperlipidemia5. Individuals with ALD have been shown to be as metabolically unhealthy as subjects with NAFLD, and it is highly possible that ALD and NAFLD coexist6. In our population-based Young Finns Study (YFS) sample, we have recently observed that alcohol consumption was significantly associated with the prevalence of FL only in normal-weight individuals, whereas in overweight or obese subjects the most significant correlates of FL were body mass index (BMI) and triglycerides7. Majority of the individuals with FL in YFS (ages 34–49 years) are men (74.1%). This is to be expected, as even though overall prevalence of NAFLD has been shown to be similar between the sexes, men reach their peak prevalence of NAFLD in their forties, whilst in women the prevalence of NAFLD is still increasing and reaches its peak in their sixth decade8. Although women are known to develop ALD with lesser alcohol consumption, men in YFS consume considerable more alcohol than the women9.

MicroRNAs (miRNAs, miRs) are small non-coding RNAs that primarily regulate gene expression by binding to target mRNAs and interfering with their translation10. MicroRNAs are transcribed from DNA to primary transcripts, which are then cleaved to shorter hairpin structures, exported to cytoplasm, and further processed to form mature one-stranded miRNAs. In most cases, miRNAs repress their targets by interaction with the 3′UTR of the target mRNA, inducing a detectable change in the mRNA levels11,12. MicroRNAs can also be transported between cells and tissues via circulation. Membrane-free miRNAs stabilized by proteins11,12 and miRNAs associated with lipid vesicles can be found in blood. Circulating miRNAs have also been found in high-density lipoproteins (HDL)13,14. Circulating miRNAs have been shown to participate in cell-to-cell communication15, potentially contributing to disease progression. Humans are predicted to have 2,588 mature microRNAs (mirBase, release 21) that can control several genes, and individual miRNAs can be bound by several miRNAs—miRNAs can thus establish wide regulatory networks affecting several metabolic processes.

The development of FL has been shown to alter the miRNA profiles in the liver16,17, serum18–20, and exosomes21. MicroRNAs have also been shown to affect the regulation of cholesterol metabolism22, liver inflammation23, hepatic fibrosis24, and the development of hepatocellular carcinoma25, all associated with NAFLD and ALD26,27. Even a serum miRNA expression panel consisting of hsa-miR-122-5p, -1290, -27b-3p, and -192-5p has been suggested for clinical NAFLD diagnostics18,20. Studies have shown increased levels of circulating miR-122 in liver diseases with different etiologies26–31, indicating that it may be a potential marker for liver injury. In addition, for example the circulatory miRNAs related to inflammation, such as miR-21, -34a, -45128 and -155 have been associated with NAFLD (reviewed in refs 32 and 33). Still, many of the studies of miRNAs in FL disease have been performed with animal models. So far, miRNA profiling in humans has been done in small selected groups, and the larger studies have focused only on few specific preselected miRNAs18–21,23,26,30,34. These studies have also been performed in non-population based cohorts, and thus cannot be directly applied to general population. Therefore, wider miRNA profiling analyses with an adequate sample size and population based sampling are needed. The YFS is well-suited for this profiling, as the study participants have been extensively characterized and the population has a very low prevalence of viral hepatitis: according to the Finnish Red Cross, the prevalence of hepatitis B and C was 0.03% and 0.05%, respectively, among Finnish blood donors35.

The aims of the current study were to (i) compare blood miRNA levels between individuals with or without FL in a large population-based study cohort, (ii) investigate the association of discovered miRNAs and liver enzyme levels and explore the predictive ability of these miRNAs to detect FL over established risk factors, (iii) test the hypothesis that these miRNAs might be involved in the regulation of lipoprotein and lipid pathways connected to liver lipid accumulation by studying the association between dysregulated miRNAs and nuclear magnetic resonance (NMR) metabolomics and other characteristic previously associated with a metabolic dysfunction, (iv) further explore the mode of function of the miRNAs by analyzing the connections of these FL associated miRNAs with their predicted mRNA targets using transcriptomics analyses from the same samples. The flow and summary of the study is described in Supplementary Figure 1.

Materials and Methods

The Young Finns Study.

YFS is a multicenter follow-up study on cardiovascular risk from childhood to adulthood in Finland. The YFS was launched in 1980, when 3,596 children and adolescents (3–18 years old) participated in the baseline study35. The subjects were randomly selected from Finnish national registry among the chosen age groups and the five study districts. Thereafter, the subjects have been followed up (in 1986, 2001, 2007 and 2011) with several examinations including comprehensive risk factor assessments. The 30-year follow-up was performed in 2011, with 2,063 adults, aged 34–49 years, participating in the study. The examinations included physical measurements, blood tests, and questionnaires. Participants in the follow-up studies have been found to be more often women and older than those who dropped out, but no significant differences in risk factors have been found35. The present study has been approved by the 1st ethical committee of the Hospital District of Southwest Finland on September 21st, 2010 and by local ethical committees. All study subjects gave an informed consent and the study was conducted according to the principles of the Declaration of Helsinki. YFS samples for miRNA analysis (n = 992) were selected independently form liver status from individuals having the most comprehensive data on risk factor, metabonomic, transcriptomics, genome wide genotyping and other phenotyping
Continuous variables are presented by means with standard deviations in parentheses. abbreviations: NL = normal liver, FL = fatty liver, FLm = mild fatty liver, FLc = clearly identified fatty liver, FLAll = all subjects with fatty liver, HDL = high-density lipoprotein, LDL = low-density lipoprotein, HbA1c = glycated hemoglobin, BP = blood pressure.

**Table 1. Demographics of the subjects of Young Finns Study population with successful miRNA profiling.** Continuous variables are presented by means with standard deviations in parentheses. **Abbreviations:** NL = normal liver, FL = fatty liver, FLm = mild fatty liver, FLc = clearly identified fatty liver, FLAll = all subjects with fatty liver, HDL = high-density lipoprotein, LDL = low-density lipoprotein, HbA1c = glycated hemoglobin, BP = blood pressure.

Clinical and biochemical measurements. Weight and height were measured and BMI calculated as weight(kg)/(height(m))². Waist circumference was measured to the nearest 0.1 cm. Blood pressure was measured with a random zero sphygmomanometer.

Venous blood samples were drawn from the right antecubital vein after an overnight fast. For blood count analysis, whole blood was anticoagulated with EDTA. Blood cell parameters were measured by flow cytometric particle counting (cells) and photometry (Hb) using Sysmex XE-5000 and XT-2000i analyzers (Sysmex Corporation) with reagents provided by the manufacturer (Cellpack and Sulfolyser).

For the biochemical measurements, serum was separated, aliquoted and stored at -70 °C until analysis. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GT), glucose, cholesterol, and triglyceride concentrations were measured with ALT, AST, GT, Glucose, Cholesterol, and Triglycerides System Reagent, (Beckman Coulter Biomedical). Apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), and C-reactive protein (CRP) were determined immunoturbidimetrically (ApoA1 and B assay reagent, Orion Diagnostica and CRP Latex reagent, Beckman Coulter Biomedical). The serum triglyceride concentration was assayed using the enzymatic glycerol kinase–glycerol phosphate oxidase method (Beckman Coulter Biomedical). The serum triglyceride concentration was measured immunoturbidimetrically using the microparticle agglutination inhibition method (Fisher Diagnostics). These two concentrations were used to calculate the HbA1c percentage. All the above-mentioned assays were performed on an AU400 instrument (AU400, Olympus).

Glycated hemoglobin (HbA1c) fraction in whole blood was measured by an Abbott Architect ci8200 analyzer (Abbott Laboratories). The concentration of total hemoglobin was first determined colorimetrically, after which the concentration of HbA1c was measured immunoturbidimetrically using the microparticle agglutination inhibition method (Fisher Diagnostics). These two concentrations were used to calculate the HbA1c percentage. All the above-mentioned methods besides ALT, AST, and GT quantifications were accredited by the Finnish Accreditation Service (FINAS).

**NMR metabolomics.** A high-throughput serum NMR metabolomics platform was used for absolute quantification of serum lipids and metabolites, including lipoprotein subclass distributions, fatty acids, and various small molecules such as amino acids and glycolysis precursors (Supplementary Table 1). The analyzed 14 lipoprotein subclasses were defined based on particle size. These detailed lipoprotein subclass measurements, together with standard total lipids and apolipoproteins, provide a good basis for studying the lipid- and lipoprotein-related metabolic pathways. The NMR-based metabolic profiling has previously been used in various epidemiological studies.
and genetic studies, and details of the experimentation have been described. Data was available from all the individuals with successful miRNA profiling.

Liver ultrasonography. Ultrasound imaging and evaluation of hepatic fat in hepatic ultrasound scan images (taken in 2011) of the liver was performed using a validated protocol and Sequoia 512 ultrasound mainframes (Acuson) with 4.0 MHz adult abdominal transducers. The diagnostic evaluation of hepatic steatosis was performed visually using a standard system by a highly trained sonographer according to liver-to-kidney contrast, echogenicity, and attenuation, and was confirmed by the Agilent Small RNA Kit (Agilent). The presence of hepatic steatosis (≥fatty liver, FLAll) was assessed and the participants were further classified into subjects with clearly identifiable fatty liver (FLC) or mild fatty liver (FLM), and normal liver (NL).

RNA isolation and quality control. Whole blood (2.5 ml) was collected into Paxgene Blood RNA Tubes (PreAnalytix). The tubes were inverted 8–10 times then stored at room temperature for at least 2 hours. The tubes were then stored at −80 °C and thawed overnight before RNA isolation (both miRNA and total RNA) with a PAXgene Blood microRNA Kit (Qiagen) including the DNase Set using the Qiagar. The concentrations and purity of the RNA samples were evaluated spectrophotometrically (BioPhotometer, Eppendorf). The RNA isolation process was validated by analyzing the integrity of several RNAs with the RNA 6000 Nano Chip Kit (Agilent). The presence of the small RNA fraction was confirmed by the Agilent Small RNA Kit (Agilent).

MicroRNA expression profiling. MicroRNA expression profiling was performed with the TaqMan OpenArray MicroRNA Panel (Applied Biosystems) containing 758 microRNAs. Briefly, 100 ng of RNA was used to run both A and B pools of Megaplex (Applied biosystems) preamplification for cDNA synthesis. In the OpenArray Sample Loading Plate, 22.5 μl of each preamplified pool was mixed 1:1 with TaqMan OpenArray Real-Time PCR Master Mix. MicroRNA panels were loaded using the AccuFill System and run with the QuantStudio 12 K Flex (Applied Biosystems).

Primary data analysis was performed with Expression Suite Software version 1.0.1. As recommended by the manufacturers of the miRNA panels, RNU6, RNU44, and RNU48 were used as housekeeping small RNAs. Assays with Amplification score >1 and Cq Confidence >0.7 were accepted. Ninety-five samples were excluded due to a low number of microRNAs expressed (<200 microRNAs per sample), and in further analysis, 243 microRNAs that were expressed in at least 2/3 of the samples were included (number of microRNAs present in detectable levels in majority of the blood samples is well in line with previous similar analysis of blood tissue). The RNA quality and functionality of the TaqMan OpenArray microRNA expression panels have been validated previously. After quality control and removal of outlier miRNAs, profiling was successful on 871 samples. To correct for batch effects, the principal component analysis was performed for the miRNA expression data. The data was adjusted for 10 of the first 20 principal components from the principal component analysis.

Genome-wide expression analysis (transcriptomics). The expression levels were analyzed with an Illumina HumanHT-12 version 4 Expression BeadChip (Illumina Inc.). Utilizing the same RNA sample for both mRNA and miRNA expression profiling, 200 ng of RNA was reverse-transcribed into cDNA and biotin-UTP-labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion); 1,500 ng of cDNA was then hybridized to the Illumina HumanHT-12 v4 Expression BeadChip. The BeadChips were scanned with the Illumina iScan system. Raw illumina probe data was exported from Genomestudio and analyzed in R (http://www.r-project.org/) using the Bioconductor (http://www.bioconductor.org/) packages. The expression data was processed using nonparametric background correction, followed by quantile normalization with control and expression probes, using the nqec function in the limma package and log2 transformation. Data processing described in more detailed in ref. 43. The expression analysis was successful in 743 of the 871 samples with a miRNA expression profile.

Statistical analysis. MicroRNA expressions were compared over individuals with NL, FLM and FLC (using one-way ANOVA for normally distributed miRNAs). In order to take account of the multiple testing Bonferroni corrected p-values (p-value) were calculated and p-value < 0.05 (p < 0.00202) was considered significant. For dysregulated microRNAs fold changes (FCs) were calculated for each individual sample in comparison to the average of all individuals with NL. FL index was calculated as previously described and the levels of discovered microRNAs were correlated with this index using Spearman’s rank-order correlation and independent association was evaluated with linear regression model adjusted with age and sex.

The independent predictors of fatty liver status (FLAll vs. NL and FLM vs. NL) were researched using stepwise Akaikia information criterion (AIC) logistic regression. Three different models were used as follows: Model 1: FL status (FLAll/FLM vs. NL) predicted with discovered microRNAs only, (one by one forced in to the model); Model 2: age, sex and BMI added among explanatory variables of model 1; and fully adjusted Model 3 including all explanatory variables known to be associated with FL in YFS study. The sensitivity and specificity of the discovered microRNAs to detect individuals with FLAll or FLM was analyzed by the receiver operator curve (ROC) analysis and areas under curve (AUC) were compared between miRNAs and the liver enzymes ALT, AST and GT. To evaluate the incremental predictive value of miRNA levels
Results

Differences in blood levels of hsa-miR-122-5p and hsa-miR-885-5p between individuals with and without FL.

One-way ANOVA over liver fat status groups (NL, FLM and FLC) showed a significant increasing trend for the levels of hsa-miR-122-5p (assay number 002245, successfully profiled form 703 individuals, \( p = 7.89 \times 10^{-18} \)) and hsa-miR-885-5p (assay number 002296, successfully profiled form 868 individuals, \( p = 1.92 \times 10^{-14} \)) in individuals with either FLM (FC = 1.45, \( p = 5.52 \times 10^{-11} \)) or FLC (FC = 1.97, \( p = 4.17 \times 10^{-11} \)) (Fig. 1A). Similarly, the expression of hsa-miR-885-5p was up-regulated in individuals with FLM (FC = 1.18, \( p = 3.80 \times 10^{-4} \)) and in those with FLC (FC = 1.55, \( p = 4.30 \times 10^{-4} \)) compared to those with NL (Fig. 1B). Both, hsa-miR-122-5p and -885-5p were also up-regulated when comparing individuals with FLAll to individuals with NL, \( p = 5.22 \times 10^{-11} \) and \( p = 3.80 \times 10^{-4} \), respectively. These miRNAs were also associated with a FL, when analyzing separately in individuals with and without excess alcohol consumption (Supplementary Figure 2, One-way ANOVA over liver fat status groups; hsa-miR-122-5p \( p = 0.001 \) and \( p = 3.10 \times 10^{-12} \), respectively and hsa-miR-885-5p \( p = 0.005 \) and \( p = 0.007 \), respectively) to represent ALD and NAFLD.

There were sex miRNA interaction with hsa-miR-122-5p and hsa-miR-885-5p in respect to presence of FLAll (\( p = 2.69 \times 10^{-4} \) and 0.046, respectively). In men the association of hsa-miR-122-5p levels with FL status (NL vs. FLM vs. FLC) was in parallel with women but statistically stronger (\( p = 1.72 \times 10^{-14} \) for trend) than in women (\( p = 0.001 \)). In similar analysis for hsa-miR-885-5p levels, the differences over liver status groups was seen only in men (p = 0.001 for men and p = 0.121 for women). Hsa-miR-122-5p and -885-5p correlated with FL index (\( r = 0.281 \), \( r = 3.54 \times 10^{-14} \) and \( r = 0.105 \), \( p = 0.002 \), respectively). In linear regression model including age,
Table 2. Logistic regression models (1–3) predicting fatty liver (FLAll or FLC) with hsa-miR-122-5p or -885-5p and known risk factors and biomarkers of FL. MODEL 1: Stepwise logistic regression model predicting FL (either FLAll or FLC) with miR-122-5p or miR-885-5p (one by one forced into model). MODEL 2: Model 1 + age, sex and BMI. MODEL 3: Model 2 + alcohol consumption, waist circumference, apolipoprotein B levels, triglycerides, insulin levels, systolic blood pressure, smoking, and physical activity index. This model involves all the explanatory variables that have been previously associated with liver status in Young Finns Study [see ref. 6] excluding liver enzymes due to high correlation with studied miRNAs. Abbreviations: NL = Normal liver, FL = fatty liver, FLAll = all subjects with fatty liver, FLC = clearly identified fatty liver, miR-122 = hsa-miR-122-5p, miR-885 = hsa-miR-885-5p.

| Outcome | MODEL 1 | MODEL 2 | MODEL 3 |
|---------|---------|---------|---------|
|         | miR-122 | miR-885 | miR-122 | miR-885 | miR-122 | miR-885 |
| NL vs. FLAll |
| n   | 703     | 871     | 701     | 868     | 632     | 781     |
| p-value | 1.26 * 10^{-14} | 3.91 * 10^{-6} | 1.29 * 10^{-8} | 0.002 | 6.84 * 10^{-3} | 0.130 |
| OR   | 2.44    | 1.55    | 2.07    | 1.41    | 1.78    | 1.23    |
| 95% CI | 1.96–3.09 | 1.29–1.86 | 1.62–2.68 | 1.13–1.77 | 1.35–2.38 | 0.94–1.60 |

| NL vs. FLC |
| n   | 600     | 752     | 598     | 749     | 545     | 681     |
| p-value | 5.59 * 10^{-5} | 1.33 * 10^{-4} | 1.93 * 10^{-6} | 9.83 * 10^{-4} | 2.48 * 10^{-3} | 0.353 |
| OR   | 4.63    | 2.14    | 6.67    | 2.47    | 3.64    | 1.37    |
| 95% CI | 2.83–7.96 | 1.46–3.19 | 3.28–15.97 | 1.46–4.33 | 1.69–9.29 | 0.71–2.73 |

sex and miRNA, hsa-miR-122-5p remained a significant independent predictor of FL index (p = 4.16 * 10^{-11}, \beta = 0.227, 95% CI = 0.161–0.294 SD change in FL index per one SD increase of miRNA levels).

Hsa-miR-122-5p and -885-5p as predictors of fatty liver. In stepwise logistic regression model adjusted with age, sex and BMI hsa-miR-122-5p significantly predicted the existence of FLAll (OR = 2.07, 95% CI = 1.62–2.68, p = 1.29 * 10^{-8}) and the effect of hsa-miR-122-5p remained significant also in the fully adjusted model (OR = 1.78 95% CI = 1.35–2.38, p = 6.84 * 10^{-5}) (Table 2). In stratified analysis, with normal weight individuals (BMI ≤ 25) or overweight and obese individuals (BMI > 25) the corresponding ORs were 3.1 (95% CI = 1.54–7.0, p = 0.003) and 1.57 (95% CI = 1.17–2.13, p = 0.003), respectively (Supplementary Table 2). Similar ORs could also be seen when analyzing women and men separately (OR = 2.02, 95% CI = 1.20–3.56, p = 0.010 and OR = 1.77, 95% CI = 1.26–2.51, p = 0.001, respectively) and in moderate (<20 g alcohol per day) and excess alcohol users (≥20 g alcohol per day) (OR = 1.75, 95% CI = 1.28–2.44, p = 0.001 and OR = 2.03, 95% CI = 1.02–4.59, p = 0.059, respectively) (Supplementary Table 2).

Also hsa-miR-885-5p significantly predicted the existence of FLAll in stepwise logistic regression model adjusted with age, sex and BMI (OR = 1.41 95% CI = 1.13–1.77, p = 0.002). In the fully adjusted model (Model 3) hsa-miR-885-5p predicted FLAll when all other risk factors were added to the model (including alcohol consumption), but the addition of insulin levels abolished the significance of the predictive value of this miRNA (OR = 1.23 95% CI = 0.94–1.60, p = 0.131) (Table 2). In the sex, weight and alcohol usage stratified analysis, hsa-miR-885-5p was an independent predictor of FLAll in the model including age, BMI and sex (no included in the sex stratified analysis) but not in the fully adjusted model, with the exception of the group of subjects with excess alcohol usage where the results remained significant (OR = 2.08, 95% CI = 1.18–3.94, p = 0.016) (Supplementary Table 2).

Associations of hsa-miR-122-5p and -885-5p with serum liver enzymes. Hsa-miR-122-5p and 885-5p both correlated with the levels of ALT, AST and GT (p < 3.05 * 10^{-6} for all). Also in stepwise linear regression models including age, sex, BMI and liver status, the individual FL associated miRNAs (hsa-miR-122-5p and -885-5p one by one) predicted significantly all liver enzyme levels (Table 3).

Improvement of fatty liver prediction by hsa-miR-122-5p and -885-5p over traditional fatty liver risk factors. When comparing the utility of FL associated miRNAs in identifying individuals FLAll or FLc to commonly utilized liver enzyme levels (ALT, AST and GT), the AUC of the hsa-miR-122-5p levels from the was comparable to ALT and GT (Fig. 2A–C). Moreover, hsa-miR-122-5p outperformed AST when detecting individuals with FLc (Fig. 2A and C). In this ROC analysis when comparing AUCs of hsa-miR-885-5p levels to those of liver enzymes in detecting FLAll all liver enzymes performed better than hsa-miR-885-5p (Fig. 2B and C).

Together hsa-miR-122-5p and -885-5p slightly improved the detection of FLc beyond established risk factors alone (continuous NRI = 1.36, 95% CI = 1.06–1.67, p = 2.8 * 10^{-14}) (Table 4). Also including these miRNAs to the model predicting individuals with FLAll showed minor, but significant NRI (NRI = 0.278, 95%CI = 0.077–0.480, p = 0.0067) (Table 4). In sex stratified analysis, hsa-miR-122-5p and -885-5p slightly improved the detection of FLAll beyond established risk factors alone in both men and women separately (continuous NRI = 0.445, 95% CI = 0.068–0.821, p = 0.021 for women and continuous NRI = 0.383, 95% CI = 0.142–0.624, p = 0.0019, for men) but not in the weight stratified analysis (Supplementary Table 3).
Hsa-miR-122-5p and -885-5p as predictors of serum lipoprotein subclass components involved in lipid and lipoprotein pathways. **Statistical model:** Stepwise linear regression model predicting liver enzyme levels (one by one) with miR-122-5p or miR-885-5p (one by one forced into model), age, sex, BMI, and liver status. Abbreviations: ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, GT = gamma-glutamyltransferase, \( r \) = correlation estimate, \( p_c \) = Bonferroni corrected p-value. Betas (\( \beta \)) indicates the standard deviation (SD) change of the liver enzyme levels per increase of one SD of miRNA levels.

**Figure 2.** Receiver operating characteristic curve (ROC) analysis of the clinical utility (specificity and sensitivity) of blood levels of hsa-miR-122-5p and -885-5p in comparison to the serum levels of liver enzymes when predicting individuals with FLc (A and C) or FLAll (B and C). Abbreviations: ALT = alanine aminotransferase, AST = aspartate aminotransferase, GT = gamma-glutamyltransferase, FLAll = all subjects with fatty liver, FLc = clearly identified fatty liver.

**Table 3.** Correlation and adjusted linear regressions between hsa-miR-122-5p and -885-5p and serum liver enzyme levels. **Statistical model:** Stepwise linear regression model predicting liver enzyme levels (one by one) with miR-122-5p or miR-885-5p (one by one forced into model), age, sex, BMI, and liver status. Abbreviations: ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, GT = gamma-glutamyltransferase, \( r \) = correlation estimate, \( p_c \) = Bonferroni corrected p-value. Betas (\( \beta \)) indicates the standard deviation (SD) change of the liver enzyme levels per increase of one SD of miRNA levels.
and systolic blood pressure predicting individuals with FLAll vs NL (NL n = 108). There have also been conflicting results been detected small case-control profiling studies (n ~400 each; 4–5 miRNAs quantified)18,28. Our study also extends the data applicability to general population. Hsa-miR-122-5p is almost solely expressed in the liver, and it is the most expressed miRNA in the liver, constituting approximately 70% of liver miRNA expression46. During the progression of NAFLD, liver miR-122-5p levels were found to independently predict apoB, small VLD, IDL, and large LDL component levels, while hsa-miR-885-5p levels were inversely associated with very large HDL total lipid, phospholipid and cholesterol content. Finally, the levels of hsa-miR-885-5p correlated inversely with its predicted target mRNA levels, while hsa-miR-122-5p levels were found to independently predict apoB, small VLD, IDL, and large LDL component levels (Supplementary Table 5). OSBPL2 being the only mRNA with statistically significant negative Spearman correlation with hsa-miR-885-5p levels (after multiple testing correction), of which levels were independently predicted by hsa-miR-885-5p levels in the regression model. The majority of the 77 correlations (Spearman correlation p < 0.05) between hsa-miR-122-5p levels and its predicted target mRNA expressions levels were direct (Supplementary Table 6) and none were significant after multiple testing correction (p < 0.05 i.e. p < 0.00021). In linear regression model adjusted with age, sex, BMI, liver status, ALT, AST and GT, hsa-miR-885-5p predicted inversely (indicating a possible direct miRNA-induced target gene expression reduction) with GABA(A) receptor-associated protein (GABARAP), OSBPL2, arylsulfatase A (ARSA), and radial spoke 3 homolog (Chlamydomonas) (RSPH3) (Table 5), and OSBPL2 the only mRNA with statistically significant negative Spearman correlation with hsa-miR-122-5p levels in the prediction model of ALD also affects miR-122 expression in the human liver17. Even modest amount of alcohol consumption has been show to elevate serum miR-122 levels51. Pathological states in the liver [NAFLD16,28, ALD23, cirrhosis25, cancer26, toxicity27 and infection28] have all been shown to be associated with altered serum/plasma levels of miR-122. In support to our results associating hsa-miR-122-5p levels with liver enzyme levels, the serum levels of miR-122 have been previously shown to be associated with ALT and total cholesterol in hepatitis C patients receiving a

Table 4. Continuous net reclassification improvement (NRI) of risk stratification of fatty liver after adding hsa-miR-122-5p and hsa-885-5p to the different base models including conventional risk factors. *Statistical model: Stepwise models 1 and 2 consist of all variables associated with FL in the Young Finns Study [see ref. 6]. Variables with independent and significant prediction value were: Model 1: Alanine aminotransferase (ALT), gamma-glutamyltransferase (GT), waist circumference, insulin levels predicting individuals with FLc vs. NL (NL n = 524, FLc n = 21). Model 2: ALT, GT, waist circumference, insulin levels, and systolic blood pressure predicting individuals with FLAll vs NL (NL n = 524, FLAll n = 108). Abbreviations: AUC = area under curve, NL = normal liver, FL = fatty liver, FLAll = all subjects with FL, FLc = clearly identified fatty liver.

| Model/microRNAs | AUC | NRI (95% CI) | p-value |
|-----------------|-----|-------------|---------|
| Model 1 (predicting FLc vs. NL) | | Reference | Reference |
| +hsa-miR-122-5p | 0.986 | 0.963 (0.570–1.356) | 1.6 * 10^-4 |
| +hsa-miR-885-5p | 0.983 | 0.139 (0.296–0.575) | 0.53 |
| +hsa-miR-122-5p and hsa-miR-885-5p | 0.987 | 1.363 (1.057–1.670) | 2.8 * 10^-34 |

| Model 2 (predicting FLAll vs. NL) | | Reference | Reference |
| +hsa-miR-122-5p | 0.897 | 0.125 (0.080–0.329) | 0.23 |
| +hsa-miR-885-5p | 0.897 | 0.055 (0.151–0.262) | 1.00 |
| +hsa-miR-122-5p and hsa-miR-885-5p | 0.898 | 0.278 (0.077–0.480) | 0.0067 |

Hsa-miR-122-5p and -855-5p as predictors of their in silico predicted target mRNA levels. Correlation and adjusted (age, sex, BMI, liver status and liver enzyme levels) linear regressions between hsa-miR-122-5p and -855-5p and in silico predicted mRNA target expression levels from transcriptomics analyses are shown in Table 5. Hsa-miR-885-5p levels correlated significantly with the levels of 48 predicted mRNA targets (Supplementary Table 5). The correlations with AHNAK nucleoprotein (AHNAK), trophinin (TRO) and oxysterol binding protein like 2 (OSBPL2) were significant after multiple testing correction (p < 0.05 i.e. p < 0.00021). In linear regression adjusted for age, sex, BMI, liver status, ALT, AST and GT, hsa-miR-885-5p predicted inversely (indicating a possible direct miRNA-induced target gene expression reduction) with GABA(A) receptor-associated protein (GABARAP), OSBPL2, arylsulfatase A (ARSA), and radial spoke 3 homolog (Chlamydomonas) (RSPH3) (Table 5), and OSBPL2 the only mRNA with statistically significant negative Spearman correlation with hsa-miR-885-5p levels (after multiple testing correction), of which levels were independently predicted by hsa-miR-885-5p levels in the regression model. The majority of the 77 correlations (Spearman correlation p < 0.05) between hsa-miR-122-5p levels and its predicted target mRNA expressions levels were direct (Supplementary Table 6) and none were significant after multiple testing correction (p < 0.05 i.e. p < 0.00021). In linear regression model adjusted with age, sex, BMI, liver status, ALT, AST and GT, hsa-miR-122-5p inversely predicted eukaryotic translation initiation factor 1A, the X-linked (EIF1AX) and zinc finger CCCH-type containing 10 (ZC3H10) expressions, indicating their possible direct miRNA-induced target gene expression reduction (Table 5). In pathway analysis, miRNA targets with negative association to hsa-miR-122-5p and/or -855-5p were not enriched in any specific pathways.

Discussion

We found that elevated blood levels of hsa-miR-122-5p and -855-5p were associated with ultrasonographically detected FL in individuals with and without excess alcohol consumption in a large, population-based cohort of adults. These miRNAs highly correlated with serum liver enzyme levels and FL index. In the prediction of FLc, the levels of hsa-miR-122-5p and -855-5p in FLc were comparable to most commonly used liver enzyme levels and both hsa-miR-122-5p and -855-5p together improved risk stratification beyond established risk factors. In the metabolic analysis, hsa-miR-122-5p levels were found to independently predict apoB, small VLD, IDL, and large LDL component levels, while hsa-miR-885-5p levels were inversely associated with very large HDL total lipid, phospholipid and cholesterol content. Finally, the levels of hsa-miR-885-5p correlated inversely with its predicted target mRNA OSBPL2 expression, presenting a possible link between hsa-miR-885-5p and both HDL levels and it lipid content.

The association of hsa-miR-122-5p blood levels with FL, FL index and liver enzyme levels independently of age, sex, and BMI was shown here in a large clinically asymptomatic population. Levels of this miRNA remained as a significant predictor of FL when adjusting with known traditional risk factors of FL and the predictive value could be seen in normal weight and overweight individuals independently. This confirms the previous result where elevated miR-122 serum/plasma levels in individuals with NAFLD in comparison to those without has been detected small case-control profiling studies (p < 50)18,20,34 and two large expression analyses with selected miRNAs (n ~400 each; 4–5 miRNAs quantified)19,28. Our study also extends the data applicability to general population. hsa-miR-122-5p is almost solely expressed in the liver, and it is the most expressed miRNA in the liver, constituting approximately 70% of liver miRNA expression46. During the progression of NAFLD, liver miR-122 levels have been shown to be down-regulated in mice57,48 and humans16. There have also been conflicting results on the effects of alcohol consumption on miR-122 liver expression9,50, but it seems certain that the development of ALD also affects miR-122 expression in the human liver17. Even modest amount of alcohol consumption has been shown to elevate serum miR-122 levels51. Pathological states in the liver [NAFLD16,28, ALD23, cirrhosis25, cancer26, toxicity27 and infection28] have all been shown to be associated with altered serum/plasma levels of miR-122. In support to our results associating hsa-miR-122-5p levels with liver enzyme levels, the serum levels of miR-122 have been previously shown to be associated with ALT and total cholesterol in hepatitis C patients receiving a
nucleic acid-based miR-122 inhibitor. Priola et al. also showed significant correlation between miR-122 and AST and GT and their results indicate that miR-122 may even regulate ALT activity.

The increased levels of hsa-miR-885-5p were associated with FL. Levels of this miRNA remained an independent predictor of FL when adjusting with age, sex, BMI and most of the risk factors of FL, including alcohol consumption, but the significance was abolished when insulin levels were added to predicting model. Previous studies have indicated that hsa-miR-885-5p expression is relatively liver tissue enriched but also peripheral blood mononuclear cells have been thought to express this miRNA, indicating possible function in liver and in blood stream. In line with our results, up-regulation of plasma miR-885-5p levels has been reported in paracetamol induced liver toxicity. In contrast to a previous finding of increased levels of miR-885-5p in liver cirrhosis and hepatocellular carcinoma in a small case control study, we observed an independent and direct association between miR-885-5p and ALT, AST and GT concentrations in our wide population-based sample. In addition,
we found for the first time that hsa-miR-885-5p levels were inversely correlated with large HDL cholesterol independently of age, sex, BMI, measured liver enzyme levels and liver fat status.

Blood hsa-miR-122-5p levels were comparable to serum liver enzyme levels when identifying individuals with FL. Adding hsa-miR-122-5p and -885-5p to a model containing traditional risk factors and biomarkers of FL improved the risk stratification of FL. Although the improvement was statistically significant, it was minor and, unlike in the study by Tan et al., we did not find any predicted target mRNAs, which would have correlated negatively and significantly (after multiple testing correction) with hsa-miR-122-5p levels, indicating that this miRNA functions mainly in the liver.

| Gene ID   | Accession ID | Spearman correlation | Linear regression model* |
|-----------|--------------|----------------------|--------------------------|
|           |              | n    p-value  r    | n    p-value  β (CI(95%)) |
| Hsa-miR-122-5p |                |       |           |       |           |
| CS        | NM_004077.2  | 616   | 0.020  0.122 | 586  | 0.005  0.131 (0.041–0.222) |
| ARSB      | NM_000046.2  | 616   | 0.038  0.084 | 586  | 0.005  0.118 (0.036–0.201) |
| TRAK1     | NM_014965.2  | 616   | 0.020  0.123 | 586  | 0.009  0.122 (0.031–0.213) |
| PTP4A1    | NM_003463.3  | 616   | 0.004  0.114 | 586  | 0.011  0.113 (0.026–0.200) |
| CCDC69    | NM_015621.2  | 616   | 0.032  0.087 | 586  | 0.013  0.106 (0.023–0.190) |
| TRAK1     | NM_00104266.1| 616   | 0.032  0.086 | 586  | 0.013  0.109 (0.023–0.194) |
| CCDC43    | NM_144609.1  | 616   | 0.032  0.086 | 586  | 0.014  0.102 (0.021–0.182) |
| BCL2L2    | NM_004050.2  | 616   | 0.029  0.088 | 586  | 0.014  0.114 (0.023–0.205) |
| LCMT1     | NM_016309.1  | 616   | 0.033  0.086 | 586  | 0.014  0.105 (0.021–0.188) |
| EIF1AX    | NM_001412.3  | 616   | 0.014  −0.099 | 586  | 0.016  −0.111 (−0.201–0.021) |
| LAMCI     | NM_002293.2  | 616   | 0.016  0.097 | 586  | 0.018  0.103 (0.018–0.188) |
| SHCBP1    | NM_024745.2  | 616   | 0.047  0.080 | 586  | 0.020  0.105 (0.017–0.194) |
| SORT1     | NM_002959.4  | 616   | 0.020  0.094 | 586  | 0.031  0.099 (0.009–0.189) |
| CANX      | NM_01024649.1| 616   | 0.003  0.118 | 586  | 0.037  0.097 (0.006–0.188) |
| ZC3H10    | NM_032786.1  | 616   | 0.033  −0.086 | 586  | 0.039  −0.090 (−0.175–0.005) |
| PPAFDC1B  | NM_032483.2  | 616   | 0.044  0.081 | 586  | 0.041  0.096 (0.004–0.187) |
| GALNT10   | NM_198321.2  | 616   | 0.042  0.082 | 586  | 0.042  0.097 (0.004–0.191) |
| SUS1      | NM_022486.3  | 616   | 0.001  0.138 | 586  | 0.042  0.085 (0.003–0.167) |
| ATP5A1    | NM_004046.4  | 616   | 0.044  0.081 | 586  | 0.049  0.092 (0.001–0.184) |
| Hsa-miR-885-5p |                  |       |           |       |           |
| GABARAP   | NM_007278.1  | 740   | 0.032  −0.079 | 712  | 0.002  −0.120 (−0.194−0.046) |
| ARSA      | NM_000487.3  | 740   | 0.022  −0.084 | 712  | 0.005  −0.111 (−0.188–0.035) |
| AHNAK     | NM_024060.2  | 740   | 3.2·10^-5  0.152 | 712  | 0.013  0.094 (0.020–0.168) |
| RSPH3     | NM_031924.3  | 740   | 0.002  −0.111 | 712  | 0.014  −0.094 (−0.169–0.019) |
| OSBP2     | NM_144981.4  | 740   | 1.0·10^-4  −0.131 | 712  | 0.015  −0.095 (−0.171–0.019) |
| UGCC      | NM_199487.1  | 740   | 0.001  0.126 | 712  | 0.015  0.095 (0.019–0.172) |
| HNRNPL    | NM_001005335.1| 740   | 0.032  0.079 | 712  | 0.018  0.091 (0.016–0.166) |
| TRO       | NM_00139705.1| 740   | 4.6·10^-5  0.149 | 712  | 0.019  0.092 (0.015–0.169) |
| FCRL6     | NM_001004310.1| 740   | 0.041  0.075 | 712  | 0.022  0.093 (0.014–0.172) |
| APOL3     | NM_030644.1  | 740   | 0.001  0.118 | 712  | 0.022  0.088 (0.013–0.164) |
| PPZ1R1A   | NM_014225.3  | 740   | 0.005  0.102 | 712  | 0.028  0.087 (0.010–0.165) |

Table 5. Correlation and adjusted linear regressions between hsa-miR-122-5p and -885-5p and *in silico* predicted miRNA target expression levels from transcriptomics analysis. *Statistical model: *In silico* predicted gene target (gene ID) expression predicted with FL associated miRNAs (one by one forced in the model) adjusted with age, sex, BMI, liver status, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GT). Note: Individuals with ALT, AST or GT levels over the Finnish reference ranges were removed from the analysis.
In our transcriptomics analyses, we observed a significant inverse correlation between hsa-miR-885-5p and its target OSBPL2 expression. The OSBPL protein family has been shown to act as either lipid transporters or sterol sensors that control lipid metabolism by being involved in reverse cholesterol transport, vesicle transport, and cell signaling and by suppressing hepatic lipogenesis and VLDL production. OSBPL2 has also been previously shown to affect the metabolism of neutral lipids, possibly integrating the cellular metabolism of triglycerides with that of cholesterol. Furthermore, OSBPL2 overexpression has been shown to be associated with increased cell efflux of cholesterol, apoA1, and phosphatidyl choline vesicles to serum. Suppressing the expression of OSBPL2 may be hypothesized to be the biological pathway through which elevated hsa-miR-885-5p is associated with decreased levels of large HDL cholesterol.

A limitation of our study is that profiling miRNAs from peripheral blood poses a challenge in identifying the origin of the analyzed miRNAs, as blood contains miRNAs from circulating cells but also vesicle- and protein-bound miRNA originating from various tissues. Whole blood was selected to enable gene-expression analysis from the same sample. Although clinical diagnosis of ADL or NAFLD was not available, we had detailed information about patients lifetime alcohol consumption, liver enzyme levels, disease status and ultrasonic assessment of FL. This data was used to separate subjects with FL without significant alcohol use and FL with alcohol use. The detection of fatty liver relies on ultrasonic assessment performed as part of a scientific follow-up study, and liver biopsy (histology) was not available due to ethical reasons. According to the Finnish Red Cross, the prevalence of hepatitis B and C are 0.03% and 0.05%, respectively, among Finnish blood donors and even though we have excluded individuals with ALT, AST and GT levels over the Nordic reference ranges from transcriptomics and metabolomics analysis, we cannot exclude that some of the study subject may have had hepatitis, which could affect the results. In addition, majority of individuals with FL in our population are men. Even though sex has been included in statistical analyses as covariate and sex stratified analyses were performed, possible features specific for FL in women could have been missed due to low number of women with FL. The results cannot therefore be directly generalized to older populations with equal amounts of women and men with FL. The strengths of this study are the large, well-phenotyped population-based cohort and the availability of comprehensive molecular phenotyping by serum NMR metabolomics, which enables detailed analysis of specific biological processes through which miRNAs may exert their effects. As this work is essentially descriptive, more research is needed to shed light on the mechanism how miRNAs are released into the blood and to validate the interaction of the discovered miRNAs and their targets, for example In Vitro studies confirming the connection between hsa-miR-885-5p and OSBPL2 are needed.

In summary, we found that hsa-miR-122-5p and -885-5p are significantly up-regulated in individuals with ultrasonographically defined FL in a large Finnish population-based study cohort (YFS). The blood levels of hsa-miR-122-5p and -885-5p slightly improved FL prediction beyond established risk factors. In the metabolomics analysis, hsa-miR-122-5p levels were associated directly with levels of small VLDL, IDL, and large LDL components and hsa-miR-885-5p levels were associated inversely with extra-large HDL particle lipids and cholesterol levels. Furthermore, we found significant and inverse association between hsa-miR-885-5p and OSBPL2, a protein known to affect efflux of cholesterol.

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Author Contributions
E.R. acquired data, handled funding, performed statistical analyses, and wrote the manuscript. I.S. performed statistical analysis and reviewed the manuscript. L.-P.L. performed statistical analysis and reviewed the manuscript. J.V. participated in cohort collection and reviewed the manuscript. M.A.-K. acquired data and reviewed the manuscript. M.A.-K. acquired data and reviewed the manuscript. M.W. acquired data and reviewed the manuscript. N.K. acquired data. T.I. acquired data and reviewed the manuscript. O.R. handled funding and supervision. M.K. participated in cohort collection and reviewed the manuscript. N.H.-K. participated in cohort collection and reviewed the manuscript. R.L. handled funding and supervision. O.R handled funding, participated in cohort collection, and reviewed the manuscript. T.L. handled funding and supervision, participated in cohort collection, and contributed to the discussion, in addition to reviewing/editing the manuscript.

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