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Circulating MicroRNA-122 Is Associated With The Risk of New-Onset Metabolic Syndrome And Type-2-Diabetes

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Abstract

MicroRNA-122 (miR-122) is abundant in the liver and involved in lipid homeostasis, but its relevance to the long-term risk of developing metabolic disorders is unknown. We therefore measured circulating miR-122 in the prospective population-based Bruneck Study (n=810; survey year: 1995). Circulating miR-122 was associated with prevalent insulin resistance, obesity, metabolic syndrome, type-2 diabetes, and an adverse lipid profile. Among 92 plasma proteins and 135 lipid subspecies quantified with mass spectrometry, it correlated inversely with zinc-alpha-2-glycoprotein and positively with afamin, complement-factor H, VLDL-associated apolipoproteins, and lipid subspecies containing monounsaturated and saturated fatty acids. Proteomics analysis of livers from antagomiR-122-treated mice revealed novel regulators of hepatic lipid metabolism that are responsive to miR-122 inhibition. In the Anglo-Scandinavian Cardiac Outcomes Trial

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Duality of Interest. The Medical University of Innsbruck and King's College London filed patent applications on miRNA biomarkers.

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(ASCOT, *n*=155), 12-month atorvastatin reduced circulating miR-122. A similar response to atorvastatin was observed in mice and cultured murine hepatocytes. Over up to 15 years of follow-up in the Bruneck Study, multivariable adjusted risk ratios per 1-SD higher log miR-122 were 1.60 (95% confidence interval: 1.30-1.96; *P*<0.001) for metabolic syndrome and 1.37 (1.03-1.82; *P*=0.021) for type-2 diabetes. In conclusion, circulating miR-122 is strongly associated with the risk of developing metabolic syndrome and type-2 diabetes in the general population.

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate gene expression (1). The predominant miRNA in the liver, microRNA-122 (miR-122), has been proposed to play a central role in the regulation of lipid and glucose metabolism (2,3). Inhibition of miR-122 in mice (4,5) and non-human primates (6,7) induces fatty acid oxidation, reduces lipid synthesis, and thereby leads to lower levels of total cholesterol.

In humans, it has been suggested that miR-122 may have adverse metabolic effects and may be associated with metabolic diseases. However, as highlighted by our recent review (3), evidence from existing epidemiological studies is sparse and has important limitations. Published studies have focused on correlations with major lipids (8), whereas a breakdown into lipid subspecies would add resolution and help improve our understanding of the regulation of lipid homeostasis by miR-122. Importantly, previous studies had cross-sectional or case-control designs (3) and hence were unable to inform about long-term associations of circulating miR-122 with the development of new-onset disease outcomes over time.

To address this gap in the current literature, we conducted a series of analyses in the prospective Bruneck Study, the randomised controlled Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT), and experiments in mice and cell culture, combining lipidomics, proteomics, and miR-122 data. Our aims were four-fold. First, to assess cross-sectional correlates of circulating miR-122, including lipidomics and proteomics profiles. Second, to provide mechanistic insight into the putative regulatory function of miR-122 in lipid metabolism with animal studies of antagomiR-122 interventions and statin treatment. Third, to study the effect of statin allocation on serum miR-122 in participants of ASCOT. Fourth, to quantify the – to date unknown – associations of circulating miR-122 with the long-term risk of developing metabolic syndrome and type-2 diabetes (T2DM).

Research Design and Methods

The Bruneck Study

The Bruneck Study is a prospective, population-based study (9–13). In 1990, 1,000 individuals aged 40 to 79 years were recruited as a random sample of Bruneck inhabitants and were re-examined every 5 years since, with participation rates exceeding 90% at all surveys. The present study used the 1995 survey as baseline. Full medical records were available on clinical endpoints occurring between 1995 and 2010 (1995-2005 for metabolic syndrome) for all individuals, including those who did not participate in later evaluations or died during follow-up (100% follow-up for clinical endpoints). Metabolic syndrome was diagnosed if three out of the five following characteristics were present: (i) waist

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circumference in men ≥102 cm and women ≥88 cm; (ii) fasting triglycerides ≥150 mg/dl or on drug treatment for elevated triglycerides (fibrates and nicotinic acids); (iii) HDL cholesterol in men <40 and women <50 mg/dl or on drug treatment for reduced HDL cholesterol (fibrates and nicotinic acids); (iv) blood pressure ≥130/≥85 mmHg or antihypertensive drug treatment in a patient with a history of hypertension; and (v) fasting glucose ≥100 mg/dl or on drug treatment for elevated glucose. T2DM was diagnosed according to 1997 American Diabetes Association criteria or if the participant had a clinical diagnosis of T2DM and received anti-diabetic treatment. CVD was defined as myocardial infarction, stroke, or vascular death. Fatal and nonfatal myocardial infarction were deemed confirmed when World Health Organization criteria for definite disease status were met. Ischaemic stroke and transient ischaemic attacks were classified according to the criteria of the National Survey of Stroke. Self-report of disease was always confirmed by reviewing the participant's medical records available from their general practitioners and the Bruneck Hospital.

Risk factors were ascertained by validated standard procedures as previously described (9–13). Socioeconomic status was defined on a three-category scale (low, medium or high) on the basis of information on occupational status and educational level of the person with the highest income in the household. High socioeconomic status was assumed if the participant had ≥12 years of education or an occupation with an average monthly income ≥$2,000 (baseline salary before tax). Low socioeconomic status was defined by ≤8 years of education or an average monthly income ≤$1,000. Physical activity was assessed using the validated Baecke Score (14). Waist and hip circumferences were assessed with a plastic tape measure at the levels of the umbilicus and the greater trochanters respectively. Blood samples were taken after an overnight fast. Lipidomics profiling in plasma samples of the Bruneck cohort was performed with mass spectrometry, which allowed quantification of 135 distinct lipid species (9). HbA1c was quantified using high performance liquid chromatography (DCCT-aligned assay). The degree of insulin resistance by homeostasis model assessment (HOMA-IR) was estimated using the formula fasting plasma glucose in mmol/l × fasting serum insulin in mU/l divided by 22.5, with higher HOMA-IR values indicating higher insulin resistance (15). MiR-122 was measured in serum taken at the 1995 examination (n=810) as well as in serum and plasma taken at the 2000 examination (n=695).

The Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)

ASCOT is a double-blind randomised 2x2 factorial study of blood-pressure-lowering and lipid-lowering treatment (16–18). A total of 14,412 patients (aged 40-79 years) were randomized between 1998 and 2000 using a computer-generated optimum allocation mechanism blinded for any person involved in the undertaking of the study. Patients randomized to the lipid-lowering arm had low to moderate cholesterol levels (serum total cholesterol≤6.5 mmol/l) and were allocated atorvastatin (10mg/day) or placebo. Serum miR-122 levels were measured at baseline and 1 year after randomisation (median 13 [range 12 to 16] months) in participants of the hypertension-associated cardiovascular disease sub-study (HACVD-ASCOT) who were of European ancestry and did not have T2DM at study entry.

AntagomiR Treatment in Mice

Mice were injected intraperitoneally with antagomiR-122 and control antagomiRs (65 mg/kg; n = 5 per group) on three consecutive days as previously described (19). AntagomiRs were purchased from Fidelity Systems with the following sequences: antagomiR-122 - C*A*AACACCAUUGUCACACU*C*C*A*Chol*-T; controls - A*A*GGCAAGCUGACCUGAA*G*U*U*Chol-T. Mice were sacrificed at day 7. Liver and serum samples were harvested for analysis. Total and HDL-cholesterol were enzymatically measured using the T-Cholesterol and HDL-C Assay Kits (Wako Diagnostics) with blood samples were collected by retro-orbital venous plexus puncture after a 12h overnight fast. We measured hepatic miRNA-122 expression using Northern blot, and miR-122 and other miRNAs in liver and serum using quantitative reverse transcription real time polymerase chain reaction (qRT-PCR). Selected genes were also quantified by qRT-PCR (for details, see Supplementary material online).

Statin Treatment in Mice and Primary Murine Hepatocytes

Six-week old, female C57Bl/6 mice were injected once a day with 20mg/kg atorvastatin intraperitoneally (Sigma Aldrich, Taufkirchen, Germany) for 5 days, and were sacrificed on day 5. Serum was collected by cardiac puncture. The liver was perfused with ice-cold phosphate-buffered saline and tissue specimens from the left lower lobe were either snap frozen or placed in RNAlater (Qiagen, Hilden, Germany) until further processing. Details are provided in the Supplementary material online.

Proteomics Analyses

Targeted proteomics profiling in plasma samples of the Bruneck Study (year 2000 evaluation) was performed using multiple reaction monitoring (PlasmaDive kits, Biognosys AG), which allowed quantification of 92 proteins (for details, see Supplementary material online). During continuous operation over 2 weeks, the inter-day relative standard deviation was <20% and <5% without and with adjustment for the peak area of the authentic standard peptides, respectively.

Proteomic analysis of livers from antagomiR-treated mice was performed after an in-solution digest by liquid chromatography tandem mass spectrometry (LC-MS/MS). Differential protein expression was assessed by two methods (for details, see Supplementary material online): by spectral counting using a high mass accuracy instrument (Q-Exactive HF, ThermoFisher) and by labelling with TMT Mass Tags (ThermoFisher) using the triple-stage mass spectrometry (MS3) capability on an Orbitrap Fusion Tribrid MS (ThermoFisher). MS3 can overcome the inherent interference of more commonly used two-stage (MS2) workflows when isobaric labeling strategies are used with complex samples (20).

miR-122 Measurement using Quantitative Real-time PCR (qRT-PCR)

We measured miRNA-122 in samples of the Bruneck Study, ASCOT and statin experiments using qRT-PCR, as previously described (11,21). Briefly, total RNA was extracted using the miRNeasy kit (Qiagen, Hilden, Germany). For plasma, serum or cell culture supernatants, a fixed volume of 3μl of the 25μl RNA eluate was used as input for reverse transcription (RT)

reactions. For RNA from cells or tissue, 100ng input material was used for RT. MiRNAs were reversely transcribed using Megaplex Primer Pools (Human Pool A version 2.1 or Rodent Pool A, Life Technologies, Darmstadt, Germany) and products were further amplified using Megaplex PreAmp Primers (Primers A v2.1). Both RT and PreAmp products were stored at -20°C. Taqman miRNA assays were used to assess the expression of individual miRNAs. Diluted pre-amplification product (0.5µl) or RT product (corresponding to 0.45ng input) were combined with 0.25µl Taqman microRNA assay (20×) (Life Technologies) and 2.5µl Taqman Universal PCR Master Mix No AmpErase UNG (2×) to a final volume of 5µl. qRT-PCR was performed on an Applied Biosystems 7900HT thermocycler at 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. All samples were run in duplicate. Laboratory technicians were blinded to the participants' disease status. Relative quantification was performed using the software SDS2.2 (Life Technologies). U6 and exogenous *C. elegans* spike-in control (Cel-miR-39) were used for normalization purposes in cell and tissue experiments. For conditioned media, normalization was achieved by cultivating the same cell number in the same volume of medium with the spike-in control being used to adjust for any experimental variability in the isolation procedure.

Statistical Analysis

The statistical analysis was conducted according to a pre-specified analysis plan. MiR-122 values were log-transformed for analysis. Cross-sectional associations of miR-122 levels with other participant characteristics were quantified using Spearman correlation coefficients and linear regression models adjusted for age and sex. In the survival analysis, the principal outcomes were metabolic syndrome and T2DM, and a secondary outcome was CVD. We used Cox proportional hazard regression with updated covariates for CVD and T2DM and pooled logistic regression (22) for metabolic syndrome. Both techniques make full use of the repeat measurements of miR-122 available at the 1995 and 2000 examination. Hazard ratios and odds ratios were assumed to represent the same measure of relative risk and are collectively described as risk ratios (RR). Participants with prevalent disease were excluded from the respective analyses. Models were adjusted for age and sex, plus socio-economic status (low, medium, high), smoking (yes, no), physical activity and alcohol consumption ("multivariable model"). A sensitivity analysis further adjusted for the potential mediators/confounders body mass index and waist-hip ratio. The proportional hazards assumption for CVD and T2DM was tested using Schoenfeld residuals and was met. We investigated effect modification with formal tests for interaction across groups defined by age, sex, statin intake, and obesity. Principal analyses used significance levels of two-sided $P<0.05$. Exploratory analyses used Bonferroni-corrected P values to limit the risk of false positive results (i.e. 0.00037 for analyses of lipid subspecies; 0.00054 for proteins; 0.0042 for interaction tests). Analyses were performed using Stata software, version 12.1.

Study Approval

The Bruneck Study protocol was approved by the local ethic committee of Bolzano ('Comitato etico del comprensorio sanitario di Bolzano'; approval number 28–2010). The ASCOT protocol was approved by central and regional ethics review boards in the UK and by national ethics and statutory bodies in Ireland and the Nordic countries. Animal

experiments were approved by the Austrian authorities (licensed to A. R. Moschen No BMWF-66.011/0040-II/10b/2009) and UK authorities (licensed to Q. Xu No. PPL70/7266). The participants' written informed consent was obtained prior to their inclusion in the Bruneck and ASCOT studies.

Results

miR-122 and Major Clinical Characteristics in Participants of the Bruneck Study

We successfully quantified circulating miR-122 levels in 810 out of 826 participants of the Bruneck Study. MiR-122 levels in serum and plasma were strongly correlated (r = +0.86; 95% confidence interval: 0.84-0.88). The within-person correlation of repeat serum miR-122 measurements taken five years apart was +0.24 (0.17-0.31; Supplementary Fig. 1), which is comparable to the range previously reported for other plasma miRNAs (23). Table 1 shows baseline clinical characteristics of the Bruneck participants and their correlations with miR-122. The mean age of participants was 63 years (SD, 11) and 50% were female. Circulating miR-122 was associated with higher levels of liver enzymes, adiposity, inflammation, insulin resistance, and an adverse lipid profile (higher triglycerides and lower HDL-C) (Table 1). Participants with a diagnosis of metabolic syndrome compared to those without had 160% higher circulating miR-122 levels (P<0.001); participants with a diagnosis of T2DM compared to those without had 214% higher circulating miR-122 levels (P<0.001). No difference in circulating miR-122 levels was observed in participants with a history of CVD compared to participants without a history of CVD (P=0.969).

miR-122 and Lipidomic and Proteomic Profiles in Participants of the Bruneck Study

To provide novel insight into the complex correlation patterns of miR-122 beyond those with major clinical characteristics, we quantified cross-sectional correlations of miR-122 with lipidomic and proteomic profiles. Of the 135 distinct lipid subspecies available in the Bruneck Study (9), miR-122 showed a specific correlation with lipid subspecies comprised of monounsaturated and saturated fatty acids within the lipid classes triacylglycerols and cholesterol esters (Fig. 1*A*).

The proteomics assessment, over 4 orders of magnitude in abundance by mass spectrometry, covered 92 plasma proteins, including apolipoproteins, complement and coagulation factors (Fig. 1*B*; for full results, see Supplementary Fig. 2). Circulating miR-122 was most strongly associated with afamin (r=+0.42; P=4x10^{-30}), complement factor H (r=+0.21; P=3x10^{-8}), and zinc-alpha-2-glycoprotein (r=-0.28; P=10^{-13}). Focused analyses of apolipoproteins revealed significant positive correlations with APOB, APOC2, APOC3, APOE, and APOL1, and significant inverse correlations with APOA4 and APOD.

Treatment with AntagomiR-122 in Mice and Effect on the Hepatic Proteome

To scrutinise the role of miR-122 in the regulation of hepatic lipid metabolism, we inhibited miR-122 in mice using antagomiRs and studied consequences thereof at the miRNA, lipid, gene expression, and protein level. AntagomiR-122 led to an almost complete inhibition of miR-122 expression (Fig. 2*A*), with a secondary effect on hepatic miR-33 expression, but no effects on the expression of other miRNAs relevant to the hepatic liver metabolism,

including miR-27b and miR-148a (Fig. 2*B*). The marked inhibition of hepatic miR-122 expression by antagomiR-122 treatment was reflected in a >10-fold reduction in the mean serum levels of circulating miR-122 (P=0.032, n=10 mice per group, data not shown). Consistent with previous reports (3), antagomiR-122 treatment resulted in a reduction of total cholesterol levels (Fig. 2*C*). Gene expression in the liver was down-regulated in the liver for ATP citrate lyase (*Acly*), microsomal triglyceride transfer protein (*Mttp*), and sterol regulatory element-binding protein 1 (*Srebp1*) (Fig. 2*D*).

At the protein level, we analysed consequences of antagomiR-122 treatment using both a label-free and a TMT-labelling approach (Fig. 2*E* and Supplementary Tables 1 & 2). Eleven proteins were returned as differentially expressed in both datasets and included proteins with an apparent connection to lipid metabolism, i.e. carnitine O-palmitoyltransferase 1 (CPT1A), prolow-density lipoprotein receptor-related protein 1 (LRP1), and histidine triad nucleotide-binding protein 1 (HINT1). Few are predicted miR-122 targets (Supplementary Table 3), and the proteomics changes were not accompanied by corresponding changes in gene expression (Supplementary Fig. 3*A*). However, we observed a modest but significant downregulation of the GTPase Rab27a (Supplementary Fig. 3*B*), a key regulator of exosome release (24).

Effect of Statin Therapy on miR-122 Levels

We next assessed the effect of statin treatment on miR-122 levels. In the placebo-controlled clinical HACVD-ASCOT trial, 12-month atorvastatin treatment led not only to the expected reduction in total cholesterol and low-density lipoprotein cholesterol but also to a marked reduction in serum miR-122 levels (all $P<0.001$; Fig. 3*A*). In contrast, other miRNAs quantified in the same samples remained unchanged (data not shown).

In mice, atorvastatin treatment had only modest effects on hepatic miR-122 expression (+23%, $P=0.038$), but reduced serum miR-122 levels (-61%, $P=0.082$; Fig. 3*B*). Short-term treatment with statins in mice did not result in a reduction of total cholesterol levels (data not shown), which is in agreement with previous reports (25). Similarly, in murine primary hepatocytes, increasing doses of atorvastatin did not affect cellular miR-122 levels ($P_{trend}=0.575$), but markedly reduced miR-122 in the culture medium ($P_{trend}<0.001$; Fig. 3*C*). Thus, the inhibitory effect of atorvastatin on circulating miR-122 is independent of lipid levels and hepatic miR-122 expression.

Association of miR-122 with Development of new-onset Metabolic syndrome and T2DM

Among participants of the Bruneck free of pre-existing disease at baseline, we recorded new-onset of 136 events of metabolic syndrome and 57 events of T2DM. Age and sex-adjusted risk ratios comparing top vs. bottom third of miR-122 levels were: 2.85 (1.78-4.56; $P<0.001$) for metabolic syndrome and 2.92 (1.34-6.35; $P=0.007$) for T2DM (Fig. 4). Age- and sex-adjusted risk ratios per 1-SD higher log miR-122 were 1.59 (1.30-1.95; $P<0.001$) for metabolic syndrome and 1.39 (1.05-1.84; $P=0.021$) for T2DM. Risk ratios were virtually identical when further adjusted for socioeconomic status, smoking, physical activity, and alcohol consumption: 1.60 (1.30-1.96; $P<0.001$) for metabolic syndrome and 1.37 (1.03-1.82; $P=0.029$). Risk ratios were somewhat attenuated upon further adjustment for

body mass index and waist-hip ratio or for ln HOMA-IR. Results were broadly similar for women and men, and in subgroups according to statin intake and clinical categories of adiposity (Supplementary Fig. 4). miR-122 was not significantly associated with new-onset CVD events in the overall study (RR=1.10; 0.90-1.33; *P*=0.350), although subgroup analyses indicated a possibly stronger and significant association in participants aged <60 years compared to participants aged ≥60 years (*P* for interaction=0.006) (Supplementary Fig. 5).

Discussion

In the present study, we use a multi-dimensional 'omics approach in a population-based study to identify metabolic signatures associated with miR-122. We report a number of important and entirely novel results based on miRNA measurements in >2000 human blood samples (from the 1995 and 2000 evaluations in the Bruneck Study plus ASCOT) combined with experimental follow-up to provide a mechanistic context as summarised in Fig. 5.

First, circulating miR-122 levels are elevated in people with prevalent metabolic syndrome or T2DM and correlate strongly with lipid subspecies containing saturated and monounsaturated fatty acids. In a prospective setting, elevated serum levels of miR-122 antedate the manifestation of metabolic syndrome and T2DM, but not CVD. Second, serum levels of miR-122 positively correlate with major lipids (triglycerides, LDL- and HDL-C) in the general community and substantially decline with cholesterol-lowering statin therapy (atorvastatin 10 mg). We further corroborate this observation by *in vitro* and *in vivo* experiments demonstrating a reduction of miR-122 in the supernatant of atorvastatin-treated murine hepatocytes and in serum of atorvastatin-treated wild-type mice and confirmed miR-122 effects on enzymes involved in lipid metabolism in the liver. Overall, we provide strong evidence for circulating miR-122 being a marker of hepatic lipid metabolism.

System-wide Relations of Circulating miR-122

MiR-122, which is primarily expressed in the liver, has been suggested to regulate the expression of various genes associated with cholesterol and fatty acid metabolism (2). In mice, inhibition of miR-122 led to markedly lower plasma cholesterol levels, halted hepatic lipid synthesis, and enhanced hepatic fatty acid oxidation (4,5). Two studies in non-human primates reported similar reductions in cholesterol (6,7).

In line with these reports, our study showed that inhibition of miR-122 in mice using antagomiR-122 led to a down-regulated expression of genes implicated in lipid metabolism (Fig. 2*D*), such as ATP citrate lyase (*Acly*), microsomal triglyceride transfer protein (*Mttp*), and sterol regulatory element-binding protein 1 (*Srebp-1*) (26). This is further corroborated by the notion that miR-122 knockout mice express less *Mttp*, an essential enzyme that regulates the assembly of lipoproteins (27,28). Furthermore, our proteomic analysis of liver extracts from antagomiR-122-treated mice (Fig. 2*E*) revealed increases in carnitine O-palmitoyltransferase 1 (CPT1A), a rate-limiting enzyme of fatty acid oxidation, that was not observed at the gene expression level (Fig. 2*D*, CPT1), and prolow-density lipoprotein receptor-related protein 1 (LRP1), which plays a key role in cholesterol biosynthesis (29), HDL secretion from hepatocytes (30), and the removal of atherogenic lipoproteins, including

VLDL (31). The proteomic analysis also identified a decrease in histidine triad nucleotide-binding protein 1 (HINT1), which – based on data from *Hint-1*-deficient mice – is expected to contribute to reductions of total and esterified cholesterol (32).

These data provide mechanistic underpinning for our observation in the Bruneck Study that circulating miR-122 levels are positively associated with lipid subspecies that can be produced by hepatic *de novo* lipogenesis (Fig. 1*A*). We and others have previously shown that these lipid subspecies, comprised of saturated and monounsaturated fatty acids, are associated with a higher risk of CVD (9) and T2DM (33). We also identified strong positive correlations with apolipoproteins found on VLDL (apoB100, apoC1, apoC2, apoE), and inverse correlations with plasma apoD (present mainly in HDL) and apoA4 (a major component of HDL and chylomicrons) (Fig. 1*B*). Moreover, our comprehensive assessment of plasma proteins returned a positive correlation with afamin, which was previously linked to prevalent and new-onset metabolic syndrome and all its components (34), a positive correlation with complement factor H, a protein that binds malondialdehyde epitopes and protects from oxidative stress (35), and an inverse correlation with zinc-alpha-2-glycoprotein, an adipokine that leads to lipid degradation and higher insulin sensitivity in adipocytes (36,37).

Circulating miR-122 as Novel Biomarker

In the current study, we show – for the first time – that baseline levels of miR-122 are associated with development of metabolic syndrome and with T2DM (Fig. 4). Notably, associations did not vary by degree of adiposity, a strong determinant of cardiometabolic risk (38,39). Statin treatment decreased both lipoprotein and miR-122 release from the liver. Since miR-122 is either absent from lipoproteins, including VLDL and HDL (Supplementary Table 4), or only present at very low levels, i.e. in LDL (40), the pronounced effect of statins on circulating miR-122 levels cannot be explained by effects on plasma lipoproteins. Instead, it is probably caused by reduced secretion of liver exosomes (Fig. 5), in which miR-122 has been localized in abundance (41,42). Circulating miR-122 is undetectable in exosome-depleted serum (42). By inhibiting cholesterol synthesis, statins also modulate protein prenylation (43). This posttranslational modification promotes the membrane localisation of proteins, in particular of Rab27 proteins that control the different steps of exosome secretion (24,44). Statins may reduce circulating miR-122 levels by inhibiting the prenylation of Rab proteins and hepatic exosome secretion. The latter might constitute a novel part of the beneficial pleiotropic effects of statins. This is further corroborated by our findings in mice, demonstrating a reduction of circulating miR-122 levels after short-term treatment with atorvastatin without concomitant reduction in total cholesterol levels and reduced gene expression of Rab27a in response to antagomiR-122 treatment, the key effector GTPase that drives the exosome-release process (Supplementary Fig. 3*B*).

Strengths and Limitations

The prospective Bruneck cohort is extremely well-characterized with a 100% follow-up and high-quality ascertainment of clinical endpoints and potential confounders. MiR-122 was measured in serum and plasma. We incorporated repeat measurements of miR-122 in our

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statistical models, which is particularly important given that the within-person variability of miR-122 was high (correlation coefficient over 5 years: 0.24). In contrast to platelet-related miRNAs, which are reduced in diabetic patients (21,45), miR-122 levels showed positive associations with metabolic syndrome and T2DM and were highly correlated in serum and plasma. Expression data in the human liver would be a more direct measure, but, clearly, this is not feasible in population studies. The Bruneck Study was conducted in an entirely Caucasian population. Thus, it remains to be determined whether these findings equally apply to other ethnicities.

Conclusions

High circulating miR-122 levels correlate with complex lipids containing saturated and monounsaturated fatty acids that can be derived from hepatic *de novo* lipogenesis and an adverse metabolic profile. Inhibition of HMG-CoA reductase by atorvastatin reduces miR-122 release. Circulating miR-122 levels are associated with future development of metabolic syndrome and T2DM in the general population.

Supplementary material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136:215–233. [PubMed: 19167326]
2. Fernández-Hernando C, Ramírez CM, Goedeke L, Suárez Y. MicroRNAs in metabolic disease. Arterioscler Thromb Vasc Biol. 2013; 33:178–185. [PubMed: 23325474]
3. Willeit P, Skroblin P, Kiechl S, Fernández-Hernando C, Mayr M. Liver microRNAs: potential mediators and biomarkers for metabolic and cardiovascular disease? Eur Heart J. 2016 Epub ahead of print.
4. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 2006; 3:87–98. [PubMed: 16459310]

5. Krützfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005; 438:685–689. [PubMed: 16258535]
6. Elmén J, Lindow M, Schütz S, et al. LNA-mediated microRNA silencing in non-human primates. Nature. 2008; 452:896–899. [PubMed: 18368051]
7. Lanford RE, Hildebrandt-Eriksen ES, Petri A, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. 2010; 327:198–201. [PubMed: 19965718]
8. Gao W, He HW, Wang ZM, et al. Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. Lipids Health Dis. 2012; 11:55. [PubMed: 22587332]
9. Stegemann C, Pechlaner R, Willeit P, et al. Lipidomics profiling and risk of cardiovascular disease in the prospective population-based bruneck study. Circulation. 2014; 129:1821–1831. [PubMed: 24622385]
10. Willeit P, Kiechl S, Kronenberg F, et al. Discrimination and net reclassification of cardiovascular risk with lipoprotein(a): prospective 15-year outcomes in the Bruneck Study. J Am Coll Cardiol. 2014; 64:851–860. [PubMed: 25169167]
11. Zampetaki A, Willeit P, Tilling L, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. J Am Coll Cardiol. 2012; 60:290–299. [PubMed: 22813605]
12. Kiechl S, Wittmann J, Giaccari A, et al. Blockade of receptor activator of nuclear factor-κB (RANKL) signaling improves hepatic insulin resistance and prevents development of diabetes mellitus. Nat Med. 2013; 19:358–63. [PubMed: 23396210]
13. Willeit P, Willeit J, Brandstätter A, et al. Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. Arterioscler Thromb Vasc Biol. 2010; 30:1649–1656. [PubMed: 20508208]
14. Baecke JA, Burema J, Frijters JE. A short questionnaire for the measurement of habitual physical activity in epidemiological studies. Am J Clin Nutr. 1982; 36:936–942. [PubMed: 7137077]
15. Bonora E, Kiechl S, Willeit J, et al. Metabolic syndrome: epidemiology and more extensive phenotypic description. Cross-sectional data from the Bruneck Study. Int J Obes Relat Metab Disord. 2003; 27:1283–1289. [PubMed: 14513078]
16. Sever PS, Dahlöf B, Poulter NR, et al. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial–Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. Lancet. 2003; 361:1149–1158. [PubMed: 12686036]
17. Poulter NR, Wedel H, Dahlöf B, et al. Role of blood pressure and other variables in the differential cardiovascular event rates noted in the Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm (ASCOT-BPLA). Lancet. 2005; 366:907–913. [PubMed: 16154017]
18. Stanton A, Fitzgerald D, Hughes A, et al. An intensive phenotyping study to enable the future examination of genetic influences on hypertension-associated cardiovascular disease. J Hum Hypertens. 2001; 15(Suppl 1):S13–S18. [PubMed: 11685902]
19. Zampetaki A, Attia R, Mayr U, et al. Role of miR-195 in aortic aneurysmal disease. Circ Res. 2014; 115:857–866. [PubMed: 25201911]
20. Werner T, Sweetman G, Savitski MF, Mathieson T, Bantscheff M, Savitski MM. Ion coalescence of neutron encoded TMT 10-plex reporter ions. Anal Chem. 2014; 86:3594–3601. [PubMed: 24579773]
21. Willeit P, Zampetaki A, Dudek K, et al. Circulating microRNAs as novel biomarkers for platelet activation. Circ Res. 2013; 112:595–600. [PubMed: 23283721]
22. D'Agostino RB, Lee ML, Belanger AJ, Cupples LA, Anderson K, Kannel WB. Relation of pooled logistic regression to time dependent Cox regression analysis: the Framingham Heart Study. Stat Med. 1990; 9:1501–1515. [PubMed: 2281238]
23. Bertoia ML, Bertrand KA, Sawyer SJ, Rimm EB, Mukamal KJ. Reproducibility of Circulating MicroRNAs in Stored Plasma Samples. PLoS One. 2015; 10:e0136665. [PubMed: 26313271]
24. Ostrowski M, Carmo NB, Krumeich S, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol. 2010; 12:19–30. sup pp 1–13. [PubMed: 19966785]

25. Schonewille M, Freark de Boer J, Mele L, et al. Statins increase hepatic cholesterol synthesis and stimulate fecal cholesterol elimination in mice. J Lipid Res. 2016; 57:1455–1464. [PubMed: 27313057]
26. Menon B, Sinden J, Franzo-Romain M, Botta RB, Menon KMJ. Regulation of LH receptor mRNA binding protein by miR-122 in rat ovaries. Endocrinology. 2013; 154:4826–4834. [PubMed: 24064360]
27. Wen J, Friedman JR. miR-122 regulates hepatic lipid metabolism and tumor suppression. J Clin Invest. 2012; 122:2773–2776. [PubMed: 22820284]
28. Tsai WC, Hsu SD, Hsu CS, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest. 2012; 122:2884–2897. [PubMed: 22820290]
29. El Asmar Z, Terrand J, Jenty M, et al. Convergent Signaling Pathways Controlled by LRP1 (Receptor-related Protein 1) Cytoplasmic and Extracellular Domains Limit Cellular Cholesterol Accumulation. J Biol Chem. 2016; 291:5116–5127. [PubMed: 26792864]
30. Basford JE, Wancata L, Hofmann SM, et al. Hepatic deficiency of low density lipoprotein receptor-related protein-1 reduces high density lipoprotein secretion and plasma levels in mice. J Biol Chem. 2011; 286:13079–13087. [PubMed: 21343303]
31. Medh JD, Bowen SL, Fry GL, et al. Hepatic triglyceride lipase promotes low density lipoprotein receptor-mediated catabolism of very low density lipoproteins in vitro. J Lipid Res. 1999; 40:1263–1275. [PubMed: 10393211]
32. Beyoğlu D, Krausz KW, Martin J, et al. Disruption of tumor suppressor gene Hint1 leads to remodeling of the lipid metabolic phenotype of mouse liver. J Lipid Res. 2014; 55:2309–2319. [PubMed: 25193995]
33. Rhee EP, Cheng S, Larson MG, et al. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. J Clin Invest. 2011; 121:1402–1411. [PubMed: 21403394]
34. Kronenberg F, Kollerits B, Kiechl S, et al. Plasma concentrations of afamin are associated with the prevalence and development of metabolic syndrome. Circ Cardiovasc Genet. 2014; 7:822–829. [PubMed: 25176938]
35. Weismann D, Hartvigsen K, Lauer N, et al. Complement factor H binds malondialdehyde epitopes and protects from oxidative stress. Nature. 2011; 478:76–81. [PubMed: 21979047]
36. Garrido-Sánchez L, García-Fuentes E, Fernández-García D, et al. Zinc-alpha 2-glycoprotein gene expression in adipose tissue is related with insulin resistance and lipolytic genes in morbidly obese patients. PLoS One. 2012; 7:e33264. [PubMed: 22442679]
37. Yang M, Liu R, Li S, et al. Zinc-α2-glycoprotein is associated with insulin resistance in humans and is regulated by hyperglycemia, hyperinsulinemia, or liraglutide administration: cross-sectional and interventional studies in normal subjects, insulin-resistant subjects, and subjects with newly diagnosed diabetes. Diabetes Care. 2013; 36:1074–1082. [PubMed: 23275352]
38. Würtz P, Wang Q, Kangas AJ, et al. Metabolic signatures of adiposity in young adults: Mendelian randomization analysis and effects of weight change. PLoS Med. 2014; 11:e1001765. [PubMed: 25490400]
39. Fall T, Hägg S, Mägi R, et al. The role of adiposity in cardiometabolic traits: a Mendelian randomization analysis. PLoS Med. 2013; 10:e1001474. [PubMed: 23824655]
40. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol. 2011; 13:423–433. [PubMed: 21423178]
41. Huang X, Yuan T, Tschannen M, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC Genomics. 2013; 14:319. [PubMed: 23663360]
42. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One. 2012; 7:e30679. [PubMed: 22427800]
43. Wang CY, Liu PY, Liao JK. Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. Trends Mol Med. 2008; 14:37–44. [PubMed: 18068482]
44. Jaé N, McEwan DG, Manavski Y, Boon RA, Dimmeler S. Rab7a and Rab27b control secretion of endothelial microRNA through extracellular vesicles. FEBS Lett. 2015; 589:3182–3188. [PubMed: 26348397]

45. Elgheznawy A, Shi L, Hu J, et al. Dicer cleavage by calpain determines platelet microRNA levels and function in diabetes. Circ Res. 2015; 117:157–165. [PubMed: 25944670]

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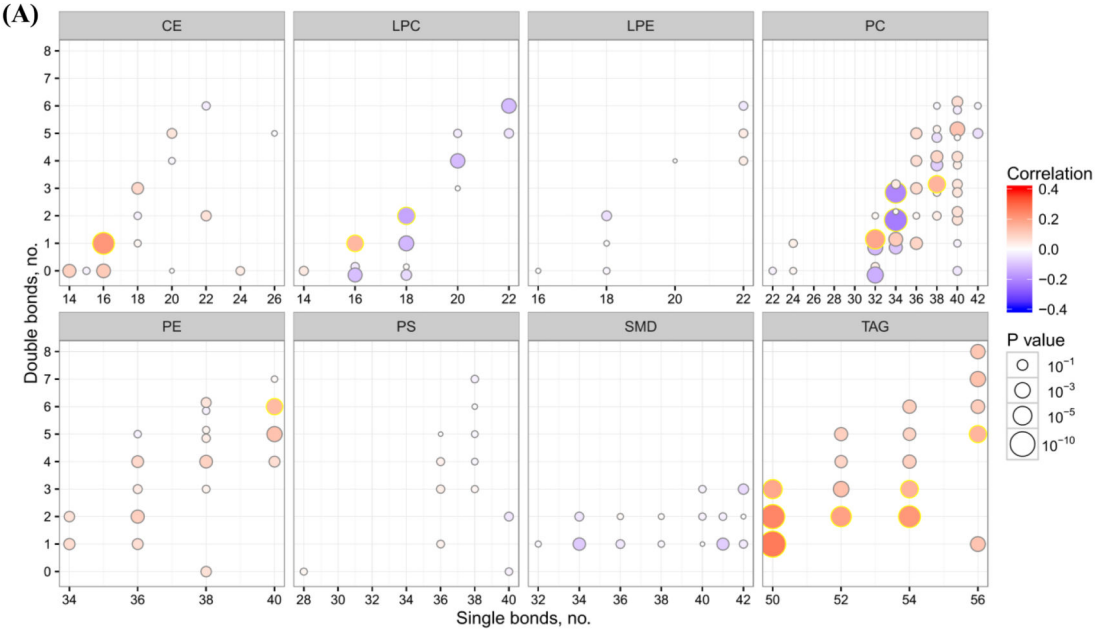
(A)



(B)

Protein	Age- and sex-adjusted Spearman correlation coefficient (95% CI) between protein and circulating miR-122	P value
Apolipoproteins		
APOA1	-0.04 (-0.12, 0.03)	0.262
APOA2	0.11 (0.03, 0.18)	0.007
APOA4	-0.16 (-0.23, -0.09)	**3 x 10^{-5}**
APOB	0.17 (0.10, 0.25)	**6 x 10^{-6}**
APOC1	0.03 (-0.05, 0.10)	0.510
APOC2	0.21 (0.13, 0.28)	**6 x 10^{-8}**
APOC3	0.17 (0.10, 0.24)	**8 x 10^{-6}**
APOD	-0.16 (-0.23, -0.08)	**4 x 10^{-5}**
APOE	0.19 (0.11, 0.26)	**1 x 10^{-6}**
APOH	0.06 (-0.01, 0.14)	0.113
APOL1	0.14 (0.06, 0.21)	**4 x 10^{-4}**
APOM	-0.02 (-0.09, 0.06)	0.626
Other proteins		
AFAM	0.42 (0.36, 0.48)	**4 x 10^{-30}**
CFAH	0.21 (0.14, 0.28)	**3 x 10^{-8}**
ZA2G	-0.28 (-0.35, -0.21)	**1 x 10^{-13}**

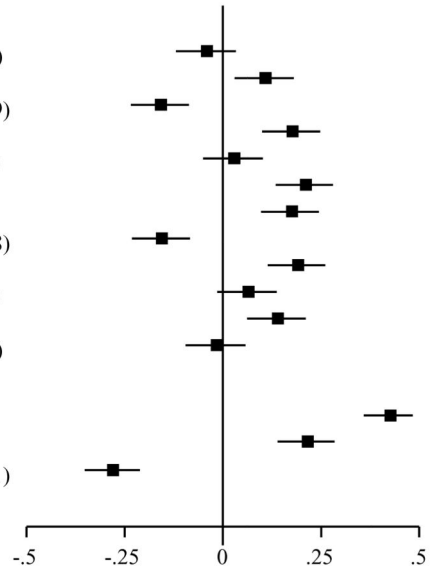


Figure 1.

Cross-sectional correlation of serum miR-122 levels with lipid subspecies (Panel A) and selected proteins related to lipid metabolism (Panel B) in the Bruneck Study. In Panel A, lipid species are arranged by lipid class in 8 panels according to the number of total carbon atoms and number of double bonds. Lipid species highlighted with a yellow halo showed statistically significant correlations after Bonferroni-correction. For better visibility, those lipid species with alkyl ether linkage are shifted upwards, whereas their alkyl-ether-free counterparts are shifted downward. In Panel B, P values significant after Bonferroni-

correction are shown in bold. The full panel of proteins are shown in Supplementary Figure 2. Abbreviations: AFAM, afamin; CE, cholesteryl ester; CFAH, complement factor H; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol; and ZA2G, zinc-alpha-2-glycoprotein.

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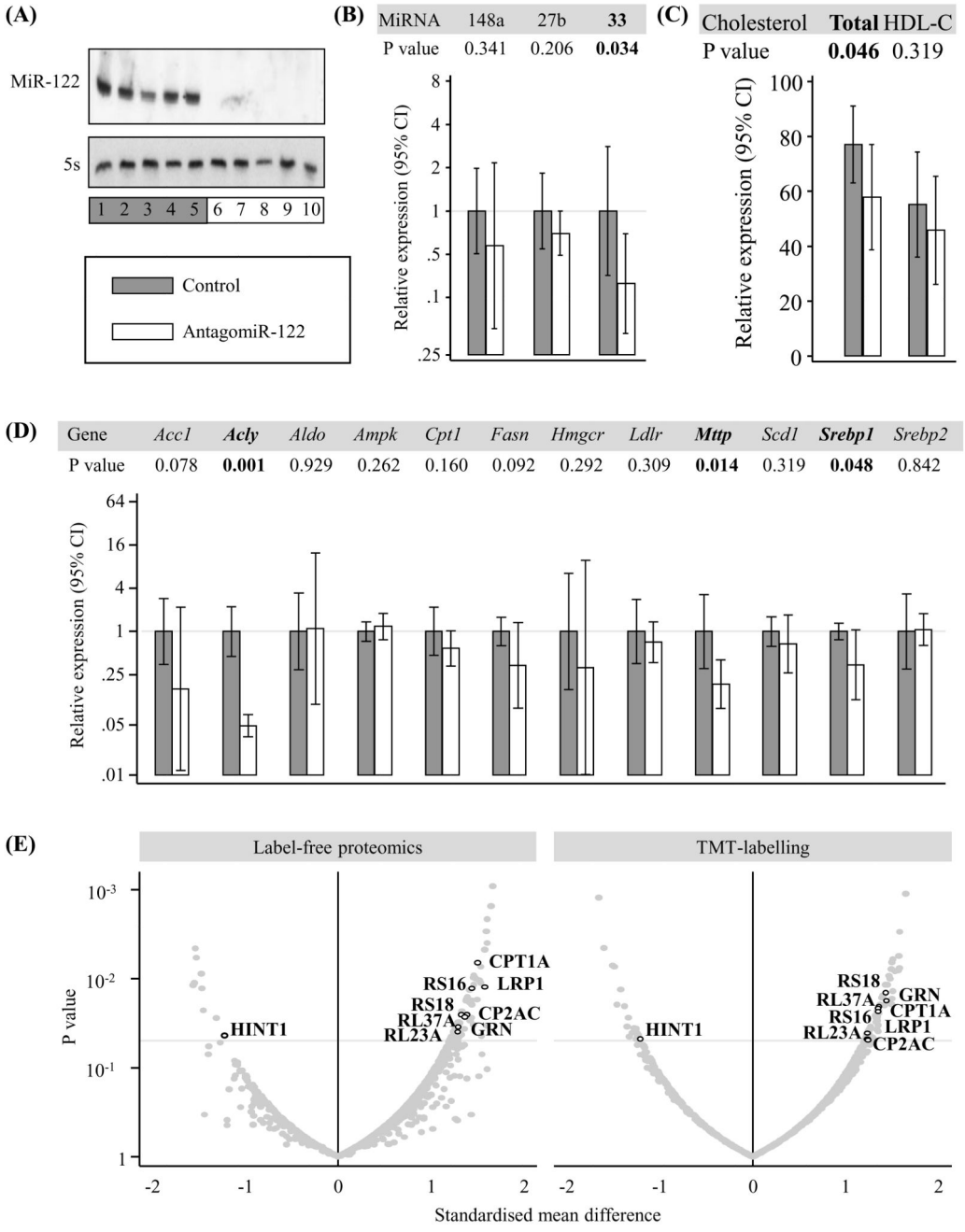

Figure 2.

Effects of antagomiR-122 injection in mice. Liver miR-122 expression was assessed by Northern blotting (Panel A); expression of other hepatic miRNAs involved in lipoprotein metabolism (Panel B); serum cholesterol (Panel C); gene expression (Panel D); and hepatic proteome profile (Panel E). Two proteomics methods were used for quantitation: a label-free method based on spectral counting and a 10-plex experiment using TMT labelling. Proteins that were returned as differentially expressed by both techniques are highlighted (for details, see Supplementary Tables 1 & 2). Abbreviations: *Acc1*, acetyl-CoA carboxylase; *Acly*, ATP

citrate lyase; *Aldo*, aldolase; *Ampk*, 5' AMP-activated protein kinase; CP2AC, cytochrome P450 2A12; *Cpt1*, carnitine palmitoyltransferase 1; CPT1A, liver isoform of carnitine O-palmitoyltransferase 1; *Fasn*, fatty acid synthase; GRN, granulins; HINT1, histidine triad nucleotide-binding protein 1; *Hmgcr*, HMG-CoA reductase; *Ldlr*, LDL receptor; LRP1, prolow-density lipoprotein receptor-related protein 1; *Mttp*, microsomal triglyceride transfer protein; RL23A, 60S ribosomal protein L23a; RL37A, 60S ribosomal protein L37a; RS16, 40S ribosomal protein S16; RS18, 40S ribosomal protein S18; *Scd1*, Stearoyl-CoA desaturase-1; and *Srebp*, sterol regulatory element-binding protein.

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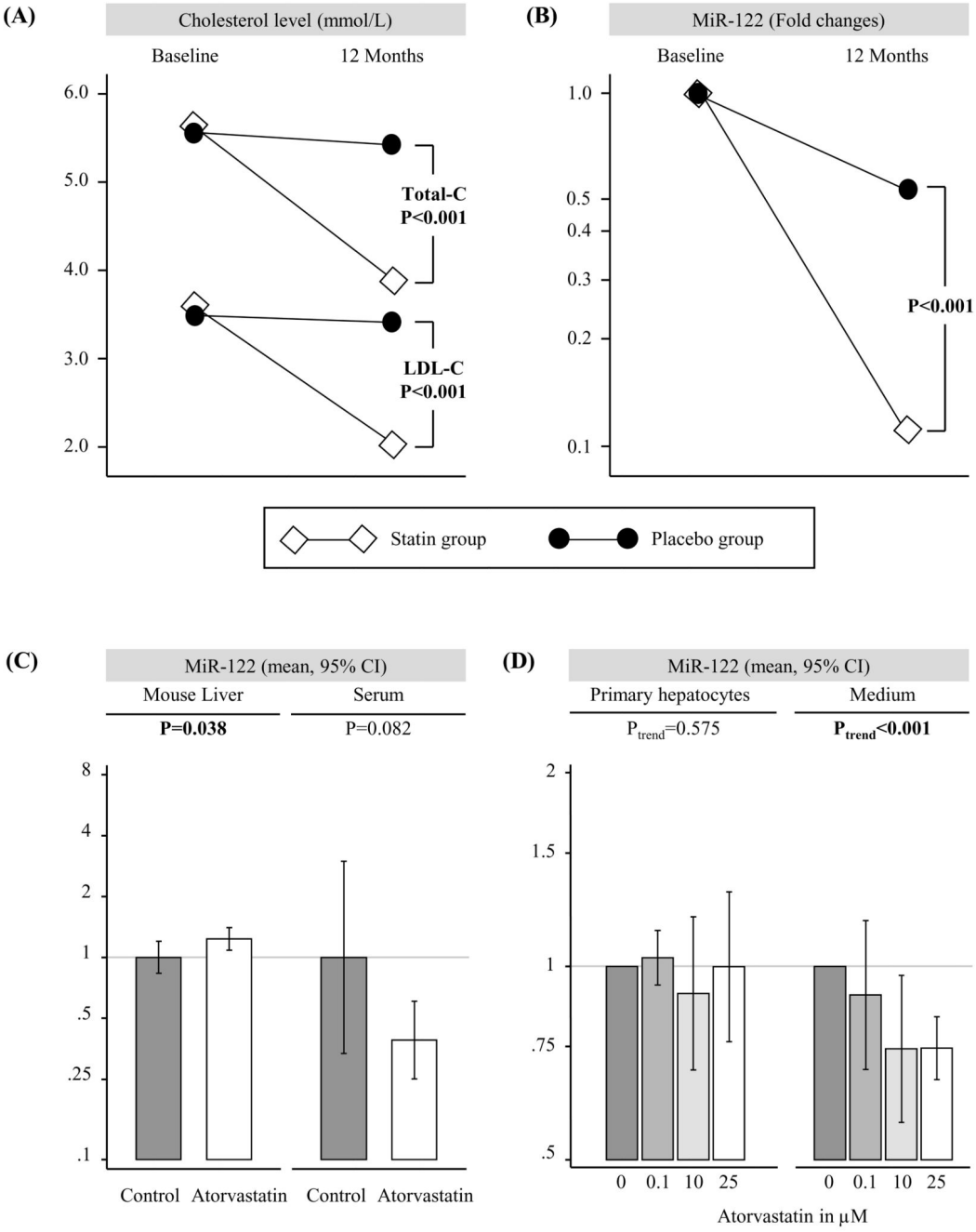

Figure 3.

Effects of atorvastatin treatment on total cholesterol, LDL cholesterol (Panel A), and serum miR-122 in ASCOT participants (Panel B), serum miR-122 in mice (Panel C), and miR-122 secretion from primary hepatocytes (Panel D).

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Outcome/ Level of adjustment	Top vs. bottom third of circulating miR-122		Per 1-SD higher circulating log miR-122	
	RR (95% CI)	P value	RR (95% CI)	P value
Metabolic syndrome (136 outcomes)				
Adjusted for age and sex	2.85 (1.78, 4.56)	**<0.001**	1.59 (1.30, 1.95)	**<0.001**
Multivariable model*	2.81 (1.76, 4.50)	**<0.001**	1.60 (1.30, 1.96)	**<0.001**
+ BMI + WHR	2.44 (1.48, 4.02)	**<0.001**	1.49 (1.19, 1.87)	**<0.001**
+ ln(HOMA-IR)	2.47 (1.53, 3.99)	**<0.001**	1.50 (1.22, 1.85)	**<0.001**
Type-2 diabetes (57 outcomes)				
Adjusted for age and sex	2.92 (1.34, 6.35)	**0.007**	1.39 (1.05, 1.84)	**0.021**
Multivariable model*	2.84 (1.30, 6.19)	**0.009**	1.37 (1.03, 1.82)	**0.029**
+ BMI + WHR	2.56 (1.16, 5.64)	**0.020**	1.31 (0.98, 1.76)	0.066
+ ln(HOMA-IR)	2.32 (1.05, 5.12)	0.037	1.26 (0.94, 1.70)	0.122

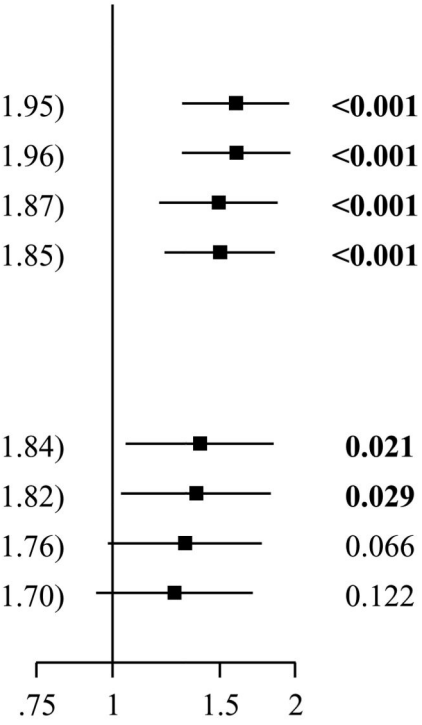

Figure 4.

Association of miR-122 with new-onset metabolic syndrome and T2DM in the Bruneck Study. *Age, sex, socio-economic status, smoking, physical activity, and alcohol consumption. Asterisks indicate level of significance: *P<0.05; **P<0.01; ***P<0.001.

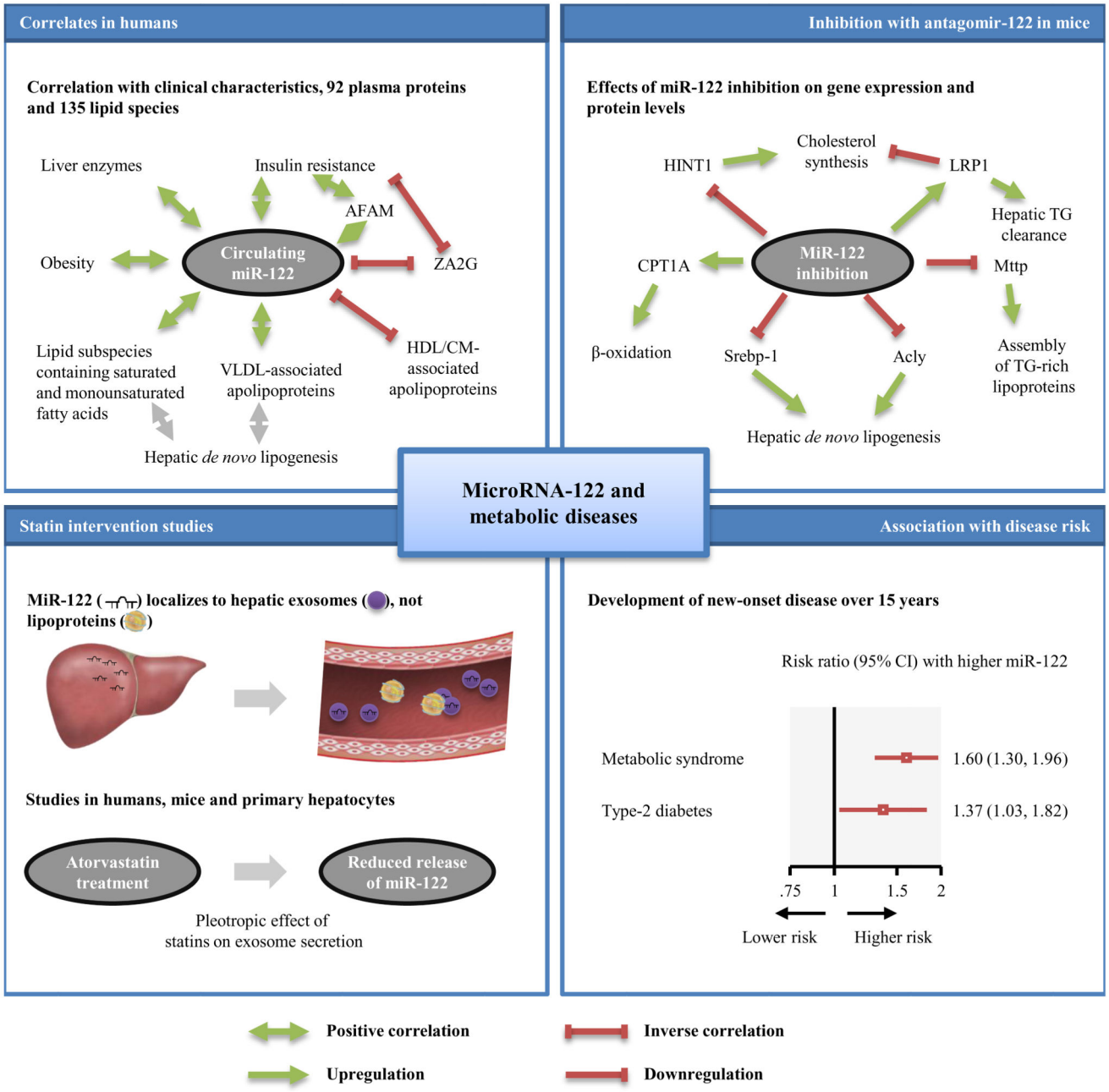

Figure 5.
Summary of the key findings.

Table 1

Baseline characteristics and cross-sectional correlates of miR-122 in the Bruneck Study.

Variable	Mean (SD) or n (%)	Age- and sex-adjusted difference in miR-122 per SD or compared to reference (95% CI)*	P value
Questionnaire-based			
Age, years	63 (11)	-3% (-19, 17%)	0.753
Female sex, n (%)	405 (50%)	+31% (-10, 89%)	0.153
Current smoker, n (%)	159 (20%)	-22% (-51, 26%)	0.314
Physical activity, Baeke score	2.3 (0.9)	+1% (-18, 24%)	0.932
Alcohol consumption, g/d	24 (31)	-1% (-21, 23%)	0.919
Statin treatment, n (%)	26 (3%)	-12% (-69, 151%)	0.808
Socioeconomic status			
Low, n (%)	494 (61%)	[Reference]	
Middle, n (%)	176 (22%)	-30% (-56, 14%)	0.150
High, n (%)	140 (17%)	+65% (-2, 177%)	0.058
Liver enzymes			
Alanine transaminase, U/l	23 (13)	+112% (76, 155%)	**<0.001**
Aspartate aminotransferase, U/l	24 (9.3)	+81% (51, 118%)	**<0.001**
Adiposity measures			
Body mass index, kg/m^2	26 (3.9)	+41% (17, 69%)	**<0.001**
Waist-hip ratio	0.93 (0.072)	+44% (17, 76%)	**<0.001**
Markers of inflammation			
Log hsCRP, mg/l	-1.7 (1.0)	+42% (17, 71%)	**<0.001**
Markers of dysglycaemia			
Fasting plasma glucose, mg/dl	102 (25)	+23% (2, 48%)	0.030
HbA1c, % [mmol/mol]	5.6 (1.8) [38 (20)]	+4% (-14, 25%)	0.704
Log HOMA-IR	1.1 (0.6)	+67% (39, 101%)	**<0.001**
Major lipids			
Total cholesterol, mg/dl	230 (43)	+19% (-1, 43%)	0.070
LDL cholesterol, mg/dl	145 (38)	+21% (1, 46%)	0.043
HDL cholesterol, mg/dl	59 (16)	-34% (-45, -21%)	**<0.001**
Log triglycerides, mg/dl	4.8 (0.5)	+62% (35, 94%)	**<0.001**