Identifying human islet microRNAs associated with donor sex, age and body mass index

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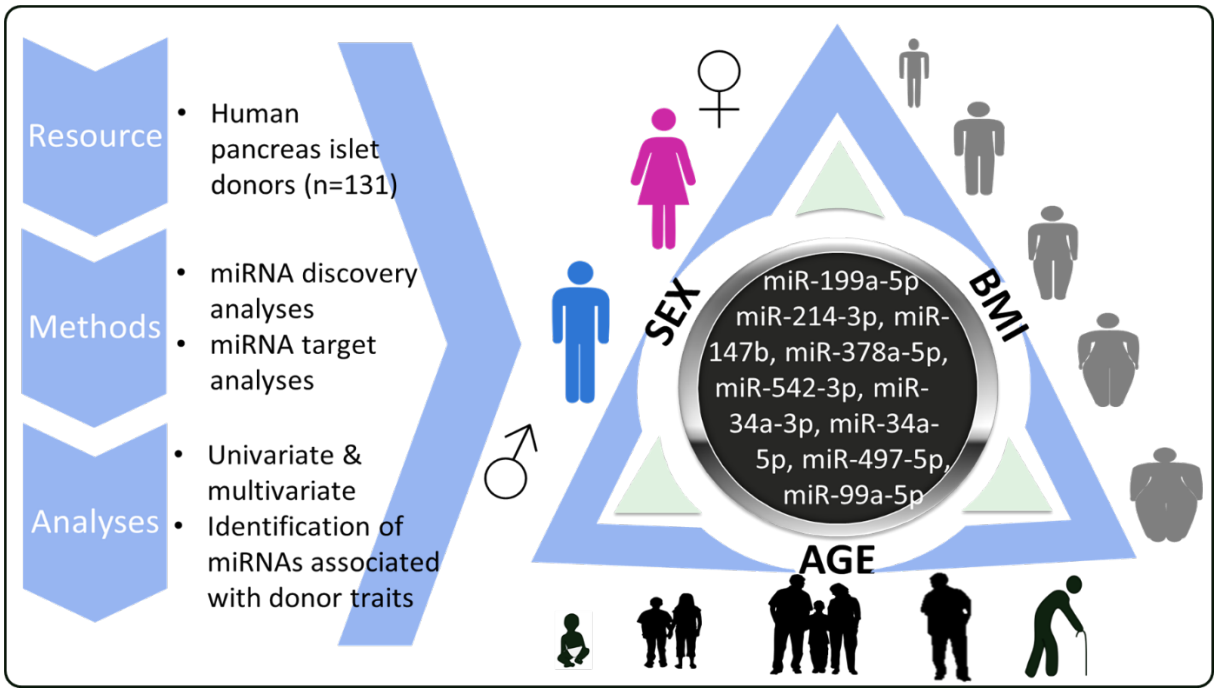

Summary

Human islets are widely used in research for understanding pathophysiological mechanisms leading to diabetes. Sex, age, and body mass index (BMI) are key donor traits influencing insulin secretion. Islet function is also regulated by an intricate network of microRNAs. Here, we profiled 754 microRNAs and 58,190 transcripts in up to 131 different human islet donor preparations (without diabetes) and assessed their association with donor traits. MicroRNA analyses identified miR-199a-5p and miR-214-3p associated with sex, age and BMI; miR-147b with sex and age; miR-378a-5p with sex and BMI; miR-542-3p, miR-34a-3p, miR-34a-5p, miR-497-5p and miR-99a-5p with age and BMI. There were 959 mRNA transcripts associated with sex (excluding those from sex-chromosomes), 940 with age and 418 with BMI. MicroRNA-199a-5p and miR-214-3p levels inversely associate with transcripts critical in islet function, metabolic regulation, and senescence. Our analyses identify human islet cell microRNAs influenced by donor traits.

Introduction

Human pancreatic islets are widely used in research to identify/validate dysregulated metabolic or apoptotic pathways leading to beta-cell failure or death in diabetes. Sex, age, and body mass index (BMI) have been indicated to influence diabetes risk [1-3].

Sex-associated differences in islet glucose response, beta-cell mass, islet oxidative stress and type 1 diabetes (T1D) incidence have been reported [4]. Testosterone, the main sex hormone in males, has been shown to enhance insulin production in mice and humans [5, 6]. DNA methylation patterns between islets from male and female donors have been linked with differences in islet gene expression, function and insulin secretion [7].

Age-related differences in DNA methylation are also reported in human islets [8]. Older (>40 years old) donor islets have lower insulin secretory response to glucose compared to those from younger donors, which may be associated with changes in beta-cell ATP generation [9]. A declining trend in beta- and non-beta-cell mass has been observed in islets from newborn to older human organ donors (aged from 0 to 79 years old) [10]. Reductions in calcium coordination, gap junction coupling, and insulin secretion dynamics were also observed in islets from older organ donors [11].

Body mass index (BMI) is a measure of obesity and an important determinant of diabetes progression [12, 13], highlighting the potential link to beta-cell function. Elevated levels of inflammatory macrophages in islets from obese individuals (BMI > 30) are contributors to insulin resistance and type 2 diabetes (T2D) susceptibility [14].

Previous studies have explored the differences in transcripts associated with donor sex, age, and BMI in human islets [7, 8, 15-17]. However, knowledge of transcriptomic variations in human islets at non-coding RNA (such as microRNA) level due to differences in sex, age and BMI of donors is lacking. This first report analyses microRNAs and their (mRNA) targets in a set of 131 human islet preparations stratified for donor traits (sex, age and BMI). Our analyses

also determine correlations of microRNAs with mRNA transcripts profiled in a sub-set (n=33) of human islets. These findings emphasize the influence of donor traits on human islet microRNA expression.

Results

Human islet microRNAs associated with sex, age and BMI.

We assessed human islet samples **(**n=101 **Figure 1A** for microRNAs and n=63 for mRNAs**; Figure 1B)** for their associations with donor sex, age, and BMI. Correlation analyses between selected microRNAs and mRNA datasets were performed in a subset (n=33) of islet donors (**Figure 1C**). A study schematic for analyses from both Australian islet isolation sites (at SVI and WIMR) is presented (**Figure 1D**).

We performed univariate analyses and adjustments for the other two traits (sex and/or age and/or BMI) on profiles of 754 discovery microRNAs in our set of human islet samples (n=101; **Figure 1A** and **Table S1**) to identify microRNAs with significant association with donor sex, age, and BMI (**Figure 2A and Table S2**). Machine learning penalized regression with bootstrapping offered validation. We identified 10 microRNAs associated with sex, 32 microRNAs associated with age and 47 microRNAs associated with BMI, based on a significance cutoff ($p<0.05$) after adjustment for the other remaining traits (**Figure 2A and Table S2**). Two microRNAs (miR-199a-5p and miR-214-3p) were associated with the three donor traits (sex, age and BMI), while several others (**Figure 2A and Table S2**) were associated with at least two of the donor traits and the importance of these microRNAs was verified using Penalized logistic/linear regression (PLR) and bootstrapping.

Both miR-199a-5p and miR-214-3p are expressed at significantly higher levels in females compared with male islets (adjusted p-value=0.032 and 0.043 respectively) and are significantly and positively correlated with BMI (adjusted p-value=0.017 and 0.039

respectively; **Table S2**). A significant increasing trend for miR-199a-5p and miR-214-3p levels was observed for islets from lean (BMI 17.9 – 24.9), overweight (BMI 25-30) and obese (BMI >30) groups (**Figure 2B and 2C**; trend p-values 0.0006 and 0.0052, respectively). Both microRNAs (miR-199a-5p and miR-214-3p) are significantly and positively correlated with age after adjustment for other traits (adjusted p-value=0.017 and 0.050 respectively, **Table S2**). When grouped by decades of age, a significant trend was observed for miR-199a-5p and miR-214-3p (**Figures 2B and C**; trend p-values 0.0384 and 0.0314, respectively).

MicroRNA-147b positively correlated (**Table S2**) with age and was detected at significantly high levels in islets from females than males (adjusted p-value=0.013, **Figure S1A**, **Table S2**). MicroRNA-378a-5p was significantly different between sex (adjusted p-value=0.029) and was also significantly (**Figure S1B**) and positively correlated with BMI (**Table S2**).

We found miR-542-3p, miR-34a-3p, miR-34a-5p, miR-497-5p and miR-99a-5p positively correlated (**Table S2**) with age and BMI. We observed that miR-34a-5p, miR-34a-3p and miR-497-5p show a significant increasing trend with age as well as with BMI (**Figure S1D-F**), while miR-99a-5p only showed a significant increasing trend for BMI (**Figure S1G**).

Target mRNAs associated with sex, age and BMI in human islets.

Next, we analysed RNA-seq profiles from human islets (n=63, **Figure 1B** and **Table S1**) for associations with donor sex, age, and BMI. We identified 959 (excluding X- and Y-linked genes, **Table S3**), 940 (**Table S4**) and 418 (**Table S5**) gene transcripts associated with sex, age, and BMI respectively (p-value<0.05), after adjustment for the other two traits examined and following FDR testing. Many of these transcripts have been reported in previous publications and associated with age, sex, and BMI; while our analyses also identified new associations between some of the transcripts and donor traits (**Table S3-S5**).

Expression quantitative Trait Loci (eQTLs) present in sex-, age- and BMI-associated genes in human islets.

Candidate genes significantly associated with sex, age or BMI (**Tables S3-S5**) were assessed on the Translational human pancreatic islet genotype tissue-expression resource (TIGER, http://tiger.bsc.es/; containing RNA-sequence and genotype profiles of 404 human islets) to identify expression quantitative trait loci (eQTLs) variations in human islets [18]. Genes such as *BARHL1*, *ADTRP* and *RASAL1* identified in our analyses have significant eQTLs variants among the islets in the TIGER database (**Table S6**).

Sex-, age- and BMI-associated microRNAs inversely correlate with mRNA transcripts critical in islet function, metabolic regulation, and senescence.

To further explore networks between sex-, age- and BMI-associated microRNAs and mRNA transcripts, correlative analysis was performed on their expression profiles in a subset of the same human islet samples (n=33; n=15 female and n=18 male, **Figure 1C** and **Table S1**). Functional interactions between microRNAs and mRNAs were assessed *in silico* (TargetScan Human ver.8). We observed significant negative and positive correlations between miR-199a-5p and miR-214-3p to target mRNAs (**Table S7 and S8**). Since majority of targeting microRNAs reduce mRNA expression [19, 20], we focussed on negatively correlated mRNAs with potential binding sites for these microRNAs. The top 10 significantly and negatively correlated mRNAs with highly conserved binding sites to miR-199a-5p are presented in **Figure 3A**, while **Figure 3B** presents the top 10 most significant negatively correlated mRNAs that contain known (albeit poorly conserved) binding sites to miR-214-3p.

MiRNA-199a-5p significantly and positively correlated to 3002 mRNAs and negatively correlated to 3136 mRNAs (**Table S7**). There were 427 genes, which negatively correlated to

miR-199a-5p with potential binding sites (for miR-199a-5p), and 74 mRNAs from those had conserved binding sites to miR-199a-5p.

MiR-214-3p, significantly and positively correlated to 3358 mRNAs and negatively to 3869 mRNAs (**Table S8**). There were 815 of the 3869 negatively correlative mRNAs with binding sites for miR-214-3p.

Most of the microRNAs associated with at least two traits (miR-147b, miR-199a-5p, -214-3p, miR-542-3p, 34a-3p, -34a-5p, miR-497-5p, miR-99a-5p and miR-378a-5p; **Figure 2A**) correlated significantly with each other (**Figure S2**). We also observed common mRNA targets for these microRNAs such as *PRDM16* and *FIGN,* that significantly and negatively correlated with both miR-199a-5p and miR-214-3p (**Figure 3A and 3B**) respectively.

Functional T2D risk genetic variants of mRNA transcripts associated with miR-199a-5p and miR-214-3p.

The top 10 mRNAs that most significantly and negatively correlated with miR-199a-5p or miR-214-3p and contained potential binding sites for these two microRNAs were assessed to identify genetic variants linked to T2D risk traits (https://t2d.hugeamp.org/). Interestingly, *FIGN* and *PRDM16 (*associated with miR-199a-5p and miR-214-3p), *PAN3* (associated with miR-199a-5p) and *THSD4* (associated with miR-214-3p) contain genetic variants, which significantly associated with BMI (**Table S9**). *TGFB2* (associated with miR-199a-5p) and *CARNS1* (associated with miR-214-3p) also contained genetic variants significantly associated with trunk fat ratio.

Sex-, age- and BMI-associated islet microRNAs are involved with transcriptional, translational, cell cycling and metabolic pathways.

Pathway over-representation analyses were performed on the predicted mRNA targets of microRNAs significantly associated with sex, age and BMI. Messenger RNA targets of the seven microRNAs (miR-199a-5p, -214-3p, -544a, let-7c-5p, -218-5p, -130b-3p, -147-3p and -138-5p) that have higher abundance in female islets, were associated with terms related to cell cycle and metabolic processes. Moreover, transcriptional and translational pathways such as mRNA processing, splicing, and translation initiation, as well as sex differentiation were also enriched (**Figure S3**). The two microRNAs (miR-378a-5p and -205-5p) with higher abundance in male islets, were over-represented in categories relating to the generation of precursor metabolites/energy and mRNA translation. Pathway analysis on mRNAs targeted by microRNAs associated with donor BMI showed involvement in metabolic processes and biological regulatory pathways. Many of the metabolic pathways (related to BMI-associated microRNAs) and cell signalling pathways were observed to be involved with donor age-associated microRNAs. Pathway analysis on miR-199a-5p and -214-3p targets revealed that their combined mRNA targets are over-represented in cell-cell signalling, biological regulation, metabolic, mRNA and translation pathways (**Figure S3**).

Discussion

With increasing shortage and costs associated with human islet isolation, most studies involving human islets assess a smaller donor set. Our analyses demonstrate that human islet microRNA (as well as their target-gene) expression may be significantly different due to donor characteristics and that these differentially expressed microRNAs, target genes associated with cell cycle/signalling, mRNA translational and metabolic pathways (**Figure S3**). Although none of the previous studies assess islet microRNAs associated with age, sex, or BMI, some studies have found an association in non-islet tissues. Several microRNAs (such as miR-214-3p, miR-199a-5p, miR-147b, miR-542-3p, miR-99a-5p and miR-378a-5p) identified in our

study, have not been previously reported to be associated with sex, age or BMI, across any other human tissues, while some microRNAs (miR-34a-5p and -3p) positively correlated with age and BMI in human vascular endothelial cells[21] and rat beta-cells [22].

In our study, two microRNAs (miR-199-5p and miR-214-3p) were associated with all three donor traits. Circulating miR-199a-5p was reported to correlate with BMI and fat mass in a childhood obesity study [23]. In addition, exosomal miR-199a-5p has been identified to be associated with hepatic lipid accumulation and fatty acid metabolism [24]. In our analysis, miR-199a-5p also correlated with genes related to obesity and inflammation such as *PRDM16*, *ADAMTSL3, ZNF300* and *TAB3* (**Table S7**). Single-nucleotide polymorphisms near *ADAMTSL3* have previously been reported to be associated with lean body mass [25], while *ZNF300* plays a role in liver lipid metabolism [26] and *TAB3* is associated with pancreatic inflammation [27].

Another previous study also identified miR-199a-5p to be associated with Klotho (an anti-aging protein) and to activate downstream signalling pathways leading to fibrosis and inflammation in kidney cells [28]. In our study, we identified the age-related gene *RBPMS* [29] to be associated with miR-199a-5p in human islets (**Table S7**). Some of the top-10 mRNAs, which showed significant negative correlation and contain conserved binding sites to miR-199a-5p are islet survival genes such as *JAG1* [30] and *TGFB2* [31] (**Figure 3A)**.

MicroRNA-214-3p has previously been suggested to play a role in metabolic function and obesity. Serum miR-214 expression levels were lower in obese (especially in insulin-resistant) individuals compared to healthy individuals [32], whilst overexpression of miR-214 in hepatocytes [33] suppressed gluconeogenesis. Here, we identified miR-214-3p to be associated with *SORCS1,* which is associated with insulin secretion [34] (**Figure 3B**), and metabolic genes such as *TPCN2* and *HFE* (**Table S8**). In human islets, *TPCN2* is important for glucagon secretion and calcium elevation [35], while *HFE* expression is associated with iron content,

insulin secretion and glucose desensitization in islets [36]. Previous studies have also implicated miR-214 in the ageing of the liver [37] and the control of mouse pancreas development [38]. In this study, we observed that miR-214-3p was associated with age-related genes such as *ABCC6* [39] (**Table S8**).

We (**Figure 2C**) and others [40] found miR-214-3p associated with donor sex in human islets. The long non-coding RNA X-inactivation specific transcript (XIST, required for inducing X-chromosome inactivation in females) has been shown to interact with miR-214-3p [41]. MiR-214-3p is also negatively correlated with the X-chromosome located gene *AR* (**Table S8**), which controls glucose/energy homeostasis and metabolic dysfunction development in various tissues (including skeletal muscle, adipose, liver and pancreas) differently between males and females [42].

MicroRNA-214 and miR-199a-2 are expressed from the same microRNA cluster on chromosome 1 and contain shared regulatory features. This may explain their common association with donor sex, age and BMI in our study. Previous studies on the miR-214/-199 cluster have identified associations with regulatory networks such as DNA hypermethylation in embryonal carcinoma cells [43] and mouse development, through interactions with Twist-1 [44], cell migration and proliferation [45]. Moreover, a study also identified the miR-199a/214 cluster as a negative regulator of brown and beige fat development and thermogenesis in adipocytes by directly targeting *PRDM16* (as part of a *PRDM16*-peroxisome PGC-1α transcriptional network) [46]. *PRDM16* is a transcription factor and histone H3 methyltransferase regulating a thermogenic gene program important for brown and beige adipocyte maintenance [47], but it is also required for islet development [48]. In our analysis, we also observed that *FIGN* was the most significantly negatively correlative gene to both miR-199a-5p and miR-214-3p (**Figure 3**). Although the function of *FIGN* is unknown in islets, *FIGN* encodes for an ATP-dependent microtubule-severing protein, and depletion of *FIGN* had

been shown to improve injured axon regeneration [49]. In human islets, miR-199a-5p and miR-214-3p may potentially bind to *FIGN* to promote cell regeneration.

Our analyses also confirmed previous studies as well as identified new target mRNAs associated with sex, age or BMI (**Table S3**). *SPESP1* expression was observed to be lower in females, consistent with a previous study [7]. Other candidates associated with donor sex and higher expression in male islets include *CDK1*, *TOP2A* and *NOX5* (**Table S3**). *NOX5* is the nearest neighbour to *SPESP1*.

A previous study had observed upregulation of *Top2a* in pancreatic islets in a model of ventromedial hypothalamic lesions [50]. *NOX5* is a key mediator of human islet insulin secretion, however, in an over-nutrition-induced environment, *NOX5* is involved in beta-cell damage [51]. Higher abundance of these genes (*TOP2A* and *NOX5*) in male islets, could be linked with the predominance of diabetes prevalence in males, as well as males being more susceptible to islet cell damage compared with females [1]. Similar sex differences in ER stress-induced genes have been recently reported [52].

Other new candidate genes, which had significantly higher expression in female islets compared to male islets, include *BARHL1*, *RPS4XP13, ART5, APOB, NLRP11* and *NLRP12*. The mRNA *BARHL1* was significantly increased in females compared with male islets. The expression of *BARHL1* is important for the migration and survival of cerebellar granule neurons [53]. Pertinent to our observation, the counterpart of *Barhl1* in *C.elegans, ceh-30* has an anti-apoptotic function in survival for sexually dimorphic cephalic male sensory neurons, regulated by sex determination pathways [54]. Some of the identified sex-associated mRNAs (such as *BARHL1* and *NOX5*) also contain putative genetic variants at their regulatory regions in islets (**Table S6**).

Our observations of multiple target genes associated with donor age are similar to a recent study, which examined the associations between the RNA-seq profiles of 37 laser-captured pancreatic islet microsections from different donors without diabetes with donor age (from 1 to 81 years old) [15]. Our analyses identified *CDKN2A*, *PAX5, SLC24A2*, *RBFOX1*, *SIX3* and *CST2* to be upregulated with age, while genes *HIST1H3E*, *FGF23*, *SOX2*, *CETP* and *BUB1B* were downregulated with increasing age. The age-associated gene *CDKN2A* has previously been studied in islets [55]; *CDKN2A* is an important cell cycle-regulating gene in pancreatic beta-cells [55] and GWAS have identified it to be significantly linked to T2D [56]. Our study also identified genes that have not been reported to be associated with age, such as *ADTRP* and *PGM5P4-AS1*.

ADTRP is involved in maintaining cell and tissue homeostasis in other tissue types (such as adipose tissue and liver) [57] and also contains putative genetic variants in islets (**Table S6**).

The analyses of islets genes associated with BMI (**Table S5**) identified genes involved in obesity, metabolism and inflammation such as *GUCY2C*, *ZNF536*, *MYBPC1*, *DOK2*, *BCL11B* and *AMY2A* that negatively correlated; and *GALNTL6, CASQ2* and *CLDN18,* which positively correlated with BMI. Our study also identified genes that have been reported to be associated with BMI (*GYG1P3* and *RASAL1*).

GYG1P3 is a pseudogene of Glycogenin 1 (*GYG1*), which is expressed across the skeletal muscle, pancreas and brain, involved in glycogen storage in muscle and glycogen synthesis in muscle and heart [58]. Chronic hyperglycaemia in a mouse model of human neonatal diabetes [59] results in marked glycogen accumulation in islets, and increased apoptosis in β-cells. *RASAL1* has been previously reported to inhibit cell growth of a hepatic tumor [60]. Putative genetic variants around the regulatory region of *RASAL1* in human islets are present (**Table S6**).

This report presents analyses of microRNA data from a large number of human islet samples, which is a major strength of this study. The study also provides diverse coverage of different ages (range:16 to 69 years), and BMI (17.9 to 57.5 kg/m^2), with a similar distribution of female and male donors. We have incorporated and provided analyses after adjustments for the other traits, multiple testing, and a machine-learning resampling approach (to eliminate sampling bias). Our study also identified multiple new candidate target genes associated with sex, age and BMI in human islets. In addition, a strength of this study is that we were able to compare microRNA and mRNA profiles in the same sub-set of human islet donors.

In our correlative analyses, we identified that the sex, age and BMI microRNAs were associated with mRNA genes involved in islet development, survival and function. We identified different metabolic (e.g. *PRMDM16, TPCN2, HFE, ADAMTSL3* and *TAB3)*, aging/senescence (e.g. *ABCC6* and *RBPMS*) or important pancreatic endocrine islet (e.g. *TGFB2, JAG1, SORCS1, TPCN2* and *HFE*) related genes associated with the sex, age and BMI microRNAs (miR-199a-5p and miR-214-3p).

Overall, our study reports microRNAs (and their targets) associated with donor traits, and also presents sub-analyses for potential microRNA-mRNA associations in human islets. Our analyses of these data provide a cautionary note to studies of specific microRNAs (and mRNA species) that may be driven by donor trait(s).

A limitation of this study is that our islet donors represent samples from all (two) human islet isolation sites within Australia. Comparison of our findings with human islets obtained from other islet isolation centres would help us understand ethnic and/or processing [61] differences. Our studies are limited to associations since methods of replicating ageing or obesity *in vitro* are difficult. Future studies at a single-cell scale will help identify specific pancreatic cell types, which are different in transcriptomic profile between certain donor traits. Single-cell RNA-seq analyses of pancreatic islets have been used to identify differences for individually

characterized pancreatic cell types associated with donor age [16, 62]. We have previously assessed microRNAs and candidate target (mRNA) gene transcripts at a single-cell resolution [63, 64]. Current technological limitations in single-cell omics for profiling both microRNA and mRNAs in human islets prevent the much-wanted datasets for single-cell miRNA and mRNA profiling. Although single-cell sequencing provides the resolution to assess cellular heterogeneity, such studies often lack the desired robustness across many donors. Future developments in analysing miRNAs and mRNAs from half-cells may enhance our observations of the association of islet microRNAs with donor traits.

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Author contributions

Author/contribution	Lab work/creation of resource	Data analysis	Manuscript writing and presentation	Conceptualization
WKMW	X	X	X	
MVJ	X	X	X	X
FC		X		
GJ		X		
AES		X		
YVC	X			
TL	X			
HET	X		X	
RCWM		X	X	
WJH	X		X	
LTD		X	X	
AAH	X	X	X	X

Declaration of interests

The authors declare no competing interests.

Tables

Excel Worksheets attached

STAR★Methods

Key Resources Table

Human islet donor details

Human islets

Isolated cadaveric human islets (**Table S1**) were obtained from donors without diabetes through the Australian Islet Transplantation Program (at Westmead Hospital, Sydney, and St Vincent's Institute, Melbourne), following the human research ethics committee (HREC) approvals from Sydney Local Health District.

Methods Details

RNA isolation, quantification and QC

Total RNA isolation and quantification for concentration and RNA integrity number (RIN) was performed as described previously [65-67]. RNA was quantified using the Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity number (RIN) was assessed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA).

Nanofluidics-based TaqMan® real-time PCR on 754 validated human microRNAs

Nanofluidic-based TaqMan® OpenArray™ human microRNA Panel was used to quantify 754 validated human microRNAs on the QuantStudio™ 12K Flex platform (Thermo Fisher Scientific, Waltham, MA) as previously detailed [68]. Details of PCR results analysis are described in our previous study [66]. MicroRNA transcript abundance was then log2 transformed for statistical analyses

Bulk RNA-sequencing and analysis

RNA-sequencing on human islet samples (GSE152111) was performed on the HiSeq4000 platform at 150 paired-end reads as detailed in previous study [67]. RNA-sequencing analysis

was carried out using the Strand Next-generation sequencing (NGS) version 2.5 software as described previously [67]. Samples were aligned to the human hg38 transcriptome and genome together (with novel splice variants) with the Ensembl genes and transcript model. Quality trimming and filter (base quality < 20 were removed) was applied after. An average of 29 million clean reads was generated. Aligned reads of all samples were quantified and normalized with the threshold normalized count set at one, using DEseq. Two of the 66 islet samples clustered with their opposite sex group (for unsupervised Euclidean average cluster analysis performed on the X and Y chromosome genes across all samples), therefore were removed for further analysis. A donor sample (H149) was duplicated in RNA-seq. The transcriptome profile of the duplicates was highly correlative (Pearson r=0.99) to each other. The average of the duplicates of the donor sample (H149) was calculated and used for analysis.

Statistical analysis

Statistical analyses were performed using R software (ver. 3.6.2-3), SPSS Statistics 27, Microsoft Excel (ver. 2016) or GraphPad Prism 8.4.1. L1-Penalized regression (Lasso) (PLR) analysis was carried out using logistic (for sex) or linear (age or BMI) regression workflow, followed by bootstrapping (n=1000 iterations) using the R package penalized (0.9.51) and glmnet (4.0-2) as described previously [65, 66].

For univariate analyse, the Kolmogorov-Smirnov test was used to check for the normal distribution of data using SPSS. F-test was performed to check for variance between the male and female in Excel. Non-parametric two-tailed Mann–Whitney U test was used to calculate the P-values, computed with no ties in R using wilcox.test() for non-normally distributed data. Two-tailed Student's (for equal variance) and Welch's (for unequal variance) t-test for normally distributed data were performed with Excel. Benjamini-Hochberg (B-H) approach was used for False Discovery Rate (FDR) multiple testing and performed with Excel. Statistical adjustment for sex, age and/or BMI were calculated with the multiple (linear) regression model

through using the lm() function in R. Kruskal-Wallis test for trend analysis was performed on GraphPad Prism. A p-value $\leq$0.05 classified as significant.

In silico analysis

The predicted potential binding sites located at and near the gene targets to the microRNAs of interest were obtained using computational web tool TargetScan Human ver. 8 [69].

Human islet Expression Quantitative Trait Loci (eQTLs) database analysis

The Translational Human Pancreatic Islet Genotype Tissue-Expression Resource (TIGER) database (http://tiger.bsc.es/) was used to search for significant expression quantitative trait loci (eQTLs) in the genes most significantly and negatively associated with sex, age and BMI microRNAs (miR-199a-5p and -214-3p).

Human T2D Genome-Wide Association Study (GWAS) database analysis. The T2D Knowledge Portal (https://t2d.hugeamp.org/) was used to search for genetic variants in the genes most significantly associated with donor sex, age and BMI in islets identified in this study.

Enrichment pathway analysis.

Pathway analyses on predicted target mRNAs of the sex, age and/or BMI-associated microRNAs (TargetScan Human ver.8) were filtered using a reference list of beta-cell expressed genes (from E-GEOD-20966). Over-representation analysis was performed using Panther, Pantherdb.org [70], and with Gene Ontology: Biological processes (GOBP) classifications using beta-cell expressed mRNAs as background set. Fishers' exact test and FDR correction for multiple testing was used.

Figures

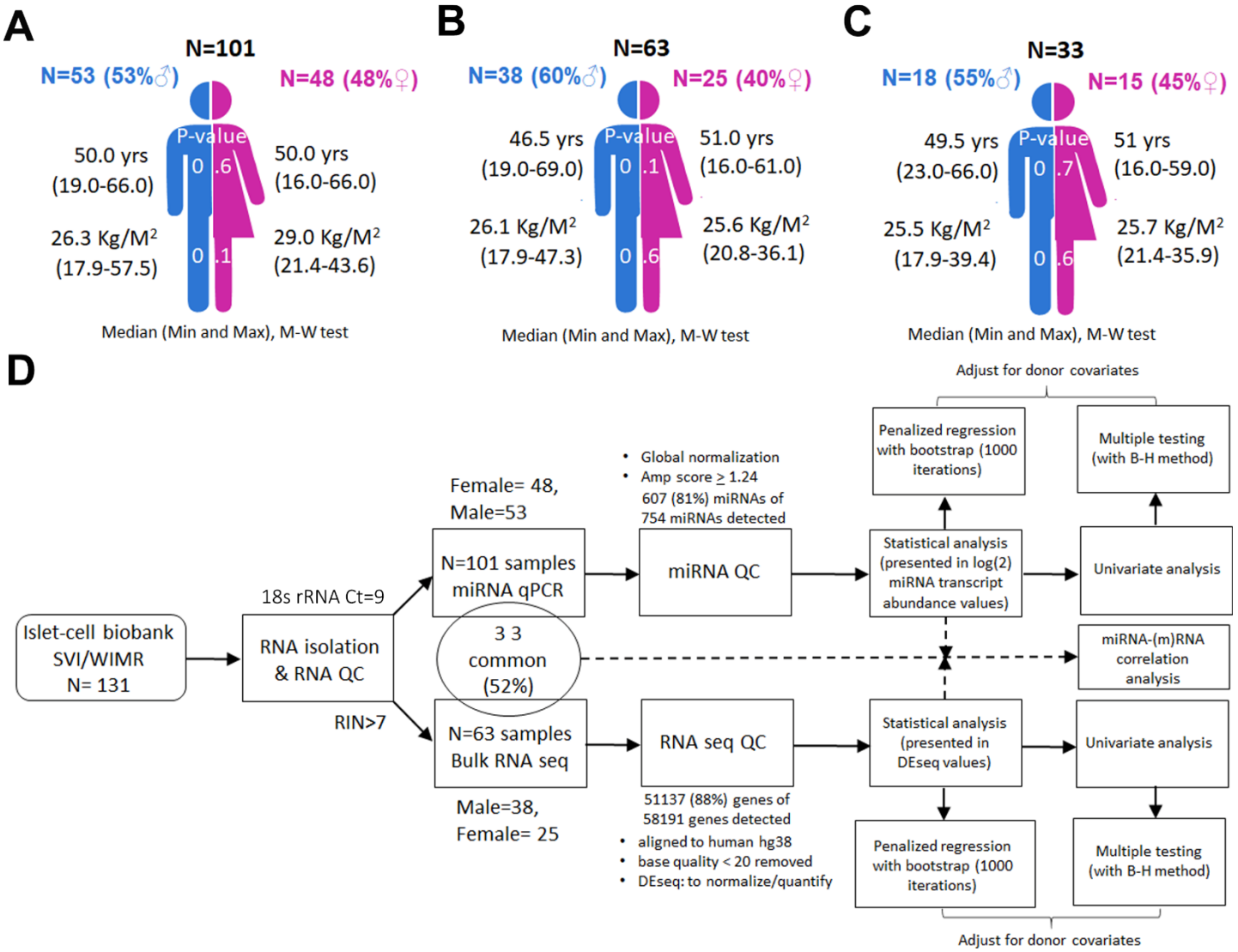
A
N=101
N=53 (53%♂)
N=48 (48%♀)
P-value
0.6
50.0 yrs
(19.0-66.0)
50.0 yrs
(16.0-66.0)
0.1
26.3 Kg/M²
(17.9-57.5)
29.0 Kg/M²
(21.4-43.6)
Median (Min and Max), M-W test
B
N=63
N=38 (60%♂)
N=25 (40%♀)
P-value
0.1
46.5 yrs
(19.0-69.0)
51.0 yrs
(16.0-61.0)
0.6
26.1 Kg/M²
(17.9-47.3)
25.6 Kg/M²
(20.8-36.1)
Median (Min and Max), M-W test
C
N=33
N=18 (55%♂)
N=15 (45%♀)
P-value
0.7
49.5 yrs
(23.0-66.0)
51 yrs
(16.0-59.0)
0.6
25.5 Kg/M²
(17.9-39.4)
25.7 Kg/M²
(21.4-35.9)
Median (Min and Max), M-W test
D
Adjust for donor covariates
Penalized regression with bootstrap (1000 iterations)
Multiple testing (with B-H method)
Female= 48,
Male=53
Global normalization
Amp score ≥ 1.24
607 (81%) miRNAs of
754 miRNAs detected
N=101 samples miRNA qPCR
miRNA QC
Statistical analysis (presented in log(2) miRNA transcript abundance values)
Univariate analysis
18s rRNA Ct=9
Islet-cell biobank SVI/WIMR N= 131
RNA isolation & RNA QC
3 3 common (52%)
miRNA-(m)RNA correlation analysis
RIN>7
N=63 samples Bulk RNA seq
RNA seq QC
Statistical analysis (presented in DEseq values)
Univariate analysis
Male=38,
Female= 25
51137 (88%) genes of
58191 genes detected
aligned to human hg38
base quality < 20 removed
DEseq: to normalize/quantify
Penalized regression with bootstrap (1000 iterations)
Multiple testing (with B-H method)
Adjust for donor covariates

Figure 1 Study workflow. Summary of the cadaveric donor islet samples (**Table S1**) and their traits (sex, age and BMI) used in **(A)** microRNA analysis (n=101) and **(B)** for RNA-seq (n=63); **(C)** microRNA-mRNA correlative analysis (n=33). Mann-Whitney test was used to assess significant differences between male and female islets. The median, minimum and maximum range are presented for donor age (year) and BMI (kg/m^2). (**D**) Flow chart of the study design. A total of 131 human islet samples obtained from different donors without diabetes through the Australian Islet Transplantation Program (at Westmead Institute for Medical Research (WIMR), Westmead Hospital, Sydney, and the St Vincent's Institute (SVI), Melbourne) was used in this study. Samples were isolated for RNA and randomly selected for microRNA (qPCR) and/or mRNA (bulk RNA-seq) analyses. Statistical analysis of donor traits (sex, age or BMI) was performed using univariate, multiple testing (using B-H approach), adjustment to other respective traits (covariate) and machine-learning penalized regression with bootstrap analysis. Correlative analysis was performed to identify the correlation between microRNA and mRNA.

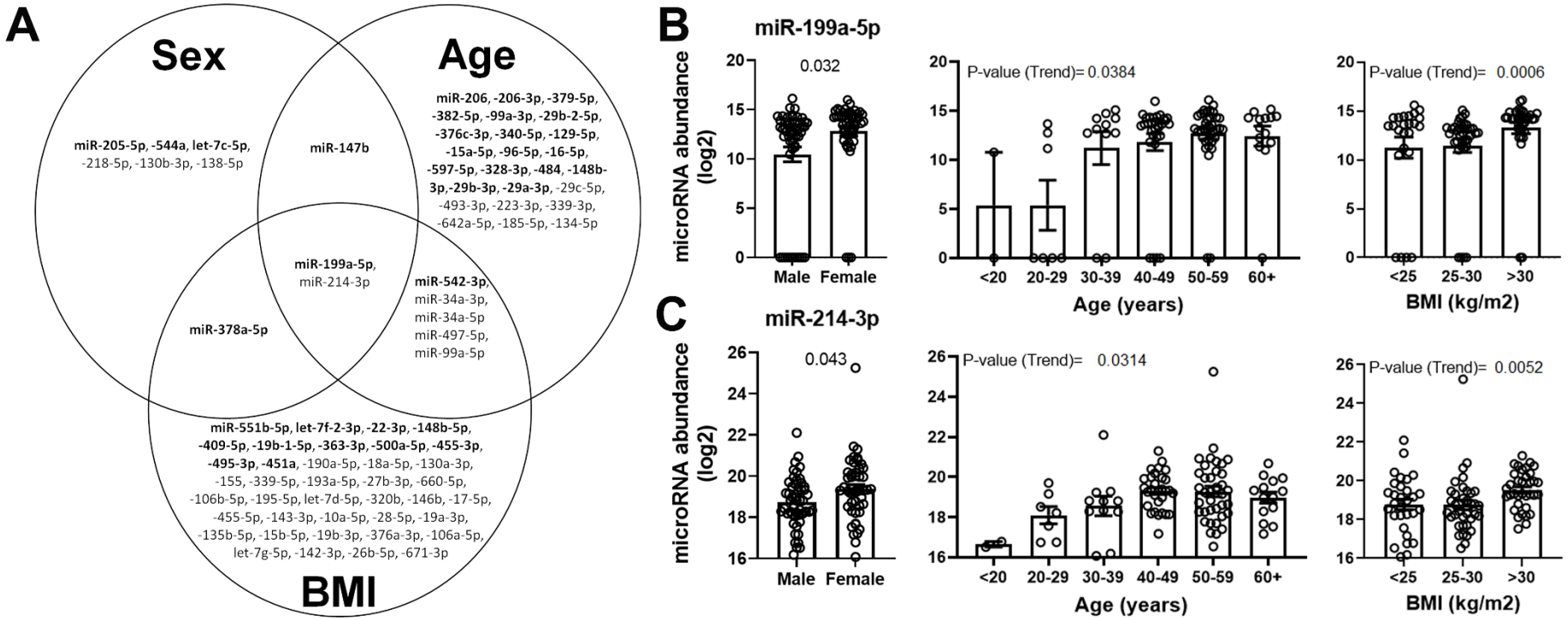

Figure 2. Human islet microRNAs associated with sex, age and BMI. (A) Venn diagram of significantly associated microRNAs with donor sex, age and/or BMI. Only the microRNAs which are detectable at least 50% of the samples and have statistical significance after adjustment (to their other two respective traits) are shown. MicroRNAs which are present also in the bootstrap (revalidation on penalized regression) analyses are in bold. MicroRNAs are ordered by highest to lowest frequency in bootstrap. Real-time TaqMan® qPCR data presented in microRNA transcript abundance (log2) for **(B)** miR-199a-5p and (**C**) miR-214-3p for sex, age and BMI. The adjusted (for age and BMI) p-value between male and female (sex) comparisons are presented. Trend analysis (using Kruskal-Wallis test) was performed to calculate p-value (trend) presented (in figures) for age or BMI. Mean $\pm$ SEM are presented.

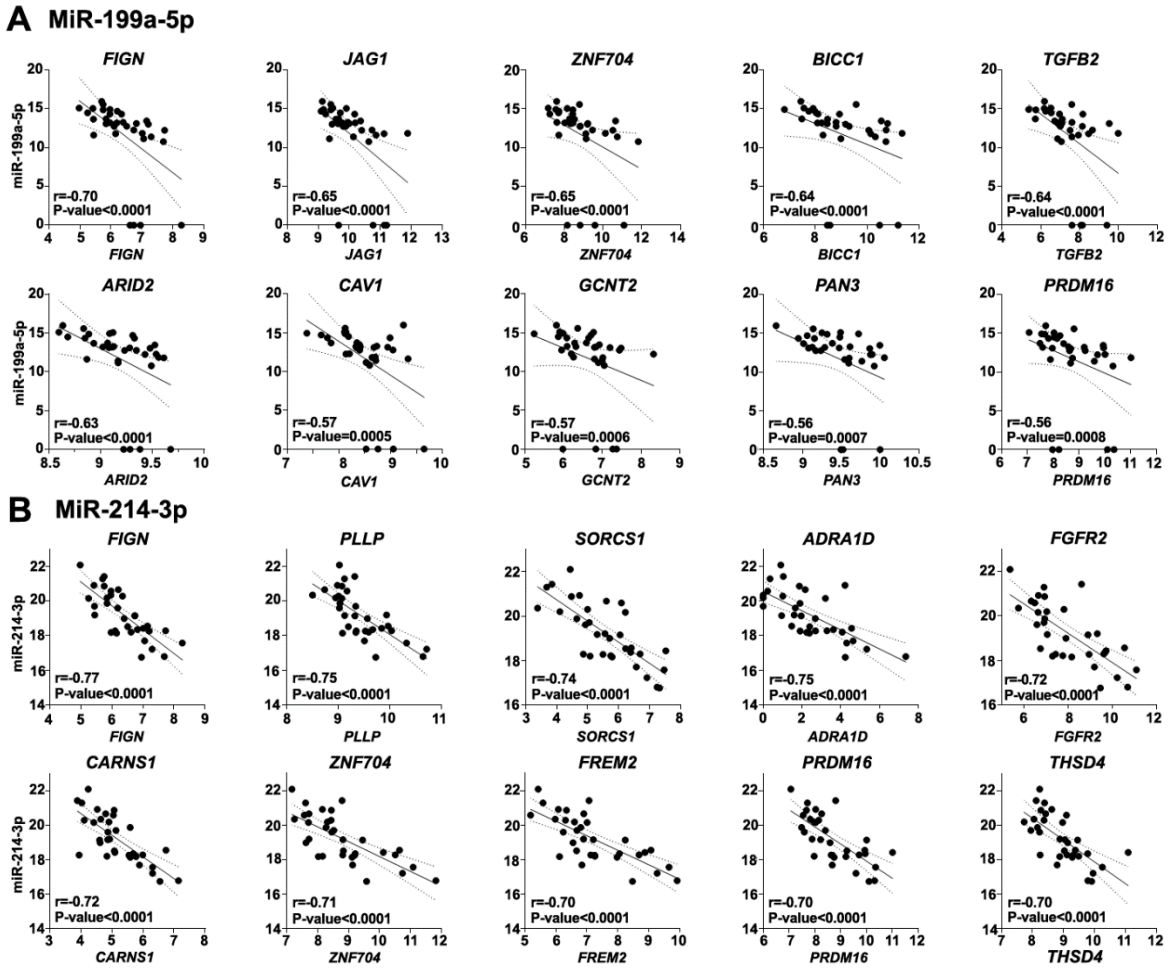

Figure 3. Sex, age and BMI microRNAs inversely correlate with critical islet function, metabolic regulation, and senescence mRNA transcripts in human islets. (**A**) MiR-199a-5p and (**B**) miR-214-3p correlation to their 10 most significantly negatively correlated genes, which contain potential conserved binding sites (related to **Table S7 and Table S8 respectively**). Presented here are the 10 most significantly negatively correlated genes with miR-214-3p. Note that miR-214-3p target genes had poor evolutionary conservation. The 95% confidence interval bands (dotted lines) and best-fit line (bold line) are presented. The Spearman coefficient (r, rho) and p-value are presented for each correlation plot.

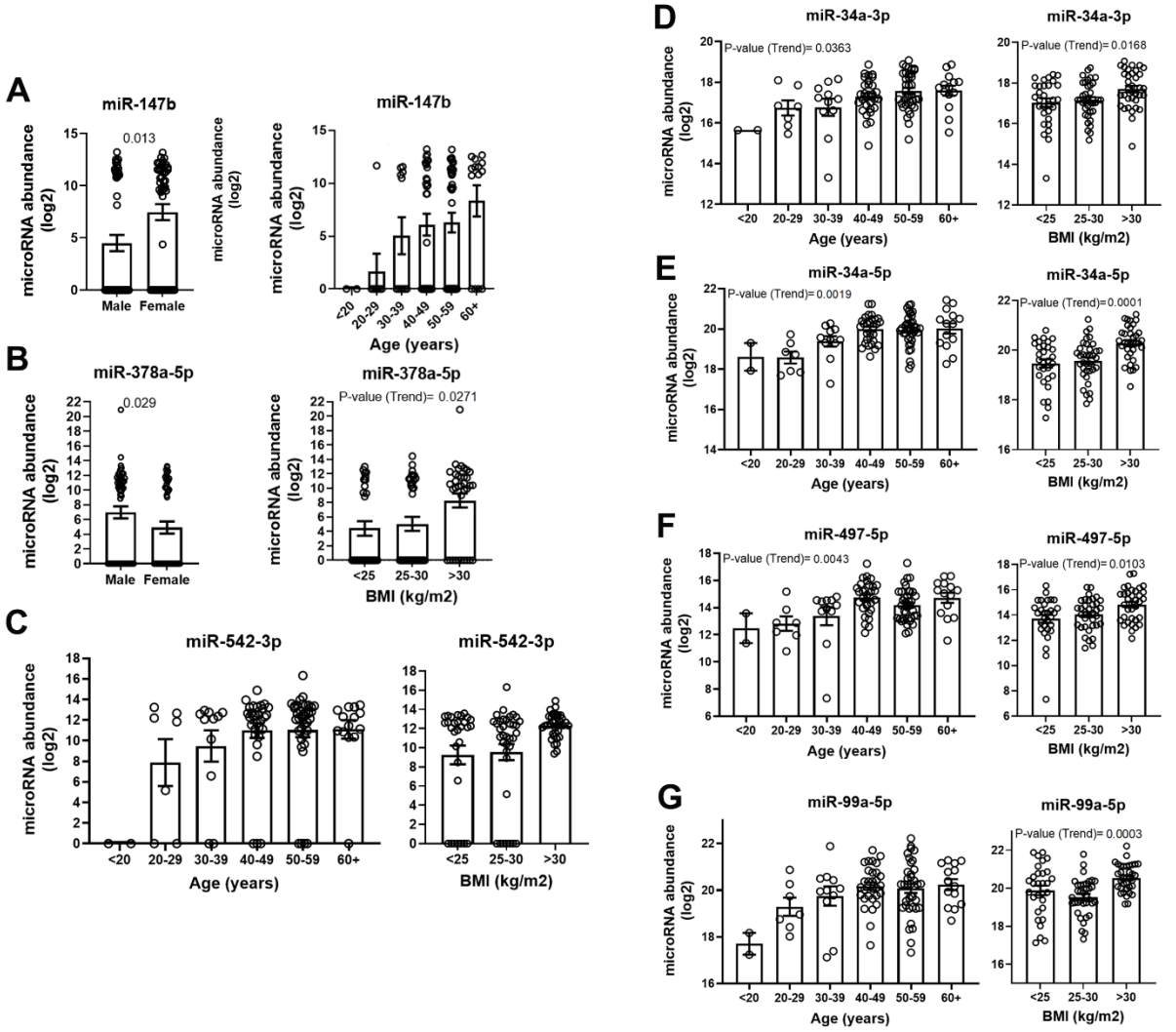

Figure S1. Human islet microRNAs associated with sex, age and BMI. MicroRNA transcript abundance (log2) for **(A)** miR-147b on sex and age; (**B**) miR-378a-5p/miR-378 on sex and BMI; (**C**) miR-542-3p, (**D**) miR-34a-3p, (**E**) miR-34a-5p, (**F**) miR-497-5p and (**G**) miR-99a-5p on age and BMI. MicroRNA quantification was performed using TaqMan rt-qPCR. The adjusted (for age and BMI) p-value between male and female (sex) comparisons are presented. Trend analysis (using Kruskal-Wallis test) was performed to calculate the p-value (trend) presented (in figures) for age or BMI. Significant trend values are provided. Mean ± SEM are presented.

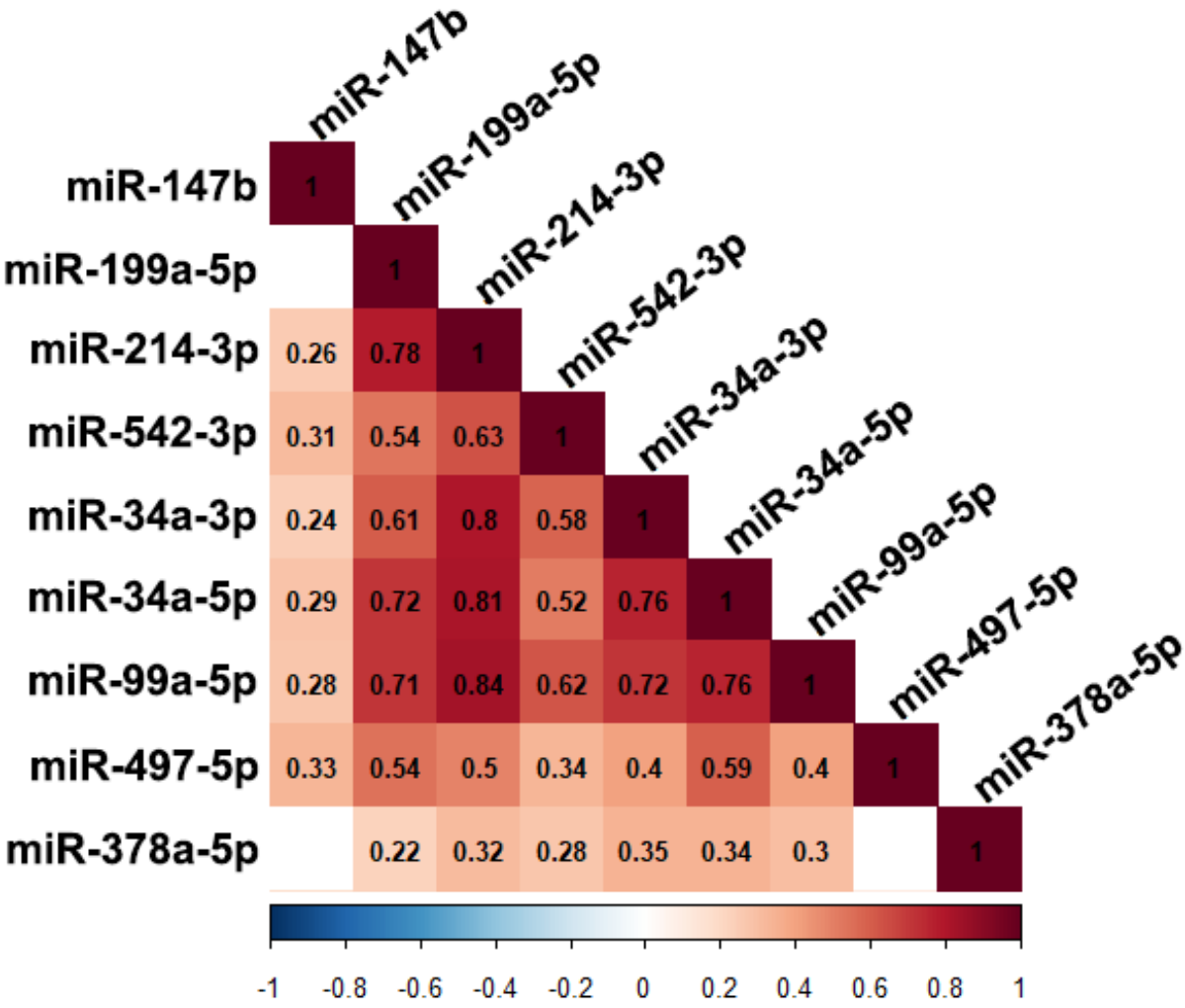

Figure S2. **Human islet sex, age and BMIC associated microRNAs associated correlation.** Correlation between sex, age and BMI associated microRNAs (miR-147b, miR-199a-5p, miR-214-3p, miR-542-3p, miR-34a-3p, miR-34a-5p, miR-99a-5p, miR-497-5p and miR-378a-5p) in human islets (n=101). The Spearman correlation rho coefficients are presented in black text in each square. Only the significant correlations (Spearman $p \leq 0.05$) are presented with a coloured (as depicted in the scale bar) in the grid displaying their Spearman correlation (rho, r) coefficient.

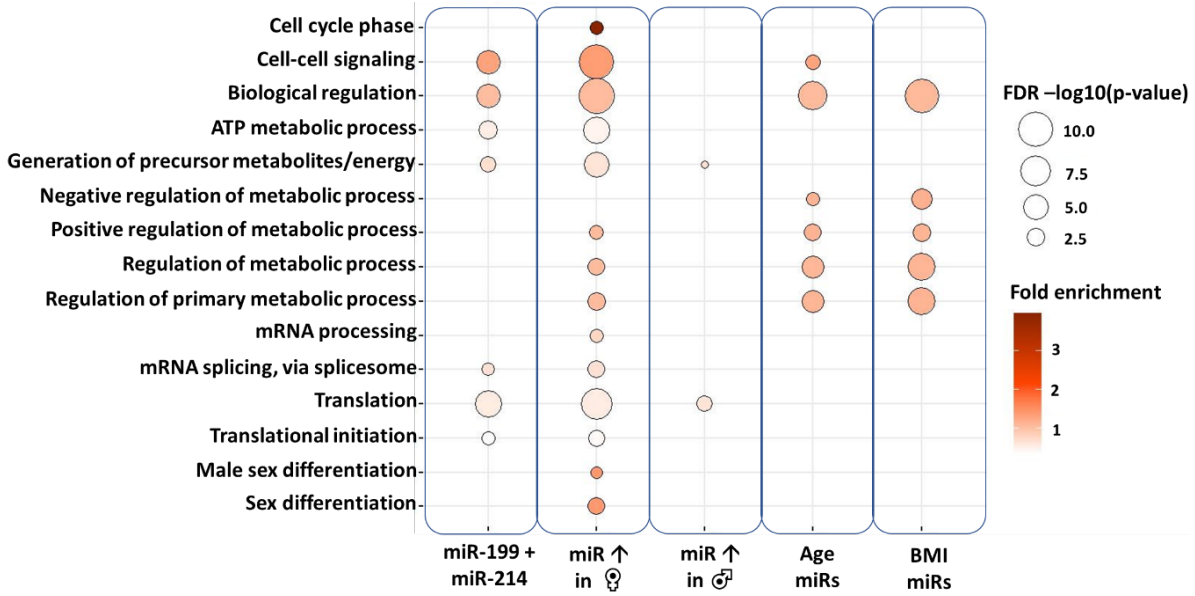

Figure S3. Sex, age and BMI-associated islet microRNAs are involved with sex differentiation, transcriptional, translational, cell cycling and metabolic enriched pathways. GOBP Enrichment pathway analyses were performed on the significant islet microRNAs associated with donor sex, age and BMI. Significant selected cell cycle and signalling, metabolic, mRNA, translational and sex differentiation pathways to the sex associated and/or BMI islet microRNAs are presented. Age miRs: represents the top 11 most significant microRNAs (after adjustment; miR-34a-5p, -34a-3p, -29b-3p, -29c-5p, -206, -147b, -379-5p, -339-3p, -642a-5p, -16-5p, -376c-3p) associated with donor age. BMI miRs: represents the top 11 most significantly microRNAs (after adjustment; miR-155, -34a-5p, -130a-3p, -18a-5p, -190a-5p, 193a-5p, 7f-2-3p, 19a-3p, -497-5p, 148b-5p and -27b-3p) associated with donor BMI. MiR ↑ in ♀: represents the microRNAs (miR-147b, -130b-3p, -218-5p, -199a-5p, -544a, -214-3p, -7c-5p and -138-5p; after adjustment) that were significantly higher in female compared to male islets. MiR ↑ in ♂: represents the microRNAs (miR-205-5p and -378a-5p; after adjustment) that were significantly higher in male compared to female islets. The size of the bubble displays the False Discovery Rate (FDR) log10 transformed p-value. The shades of red displays the fold enrichment for each pathway to the specific set of microRNAs.

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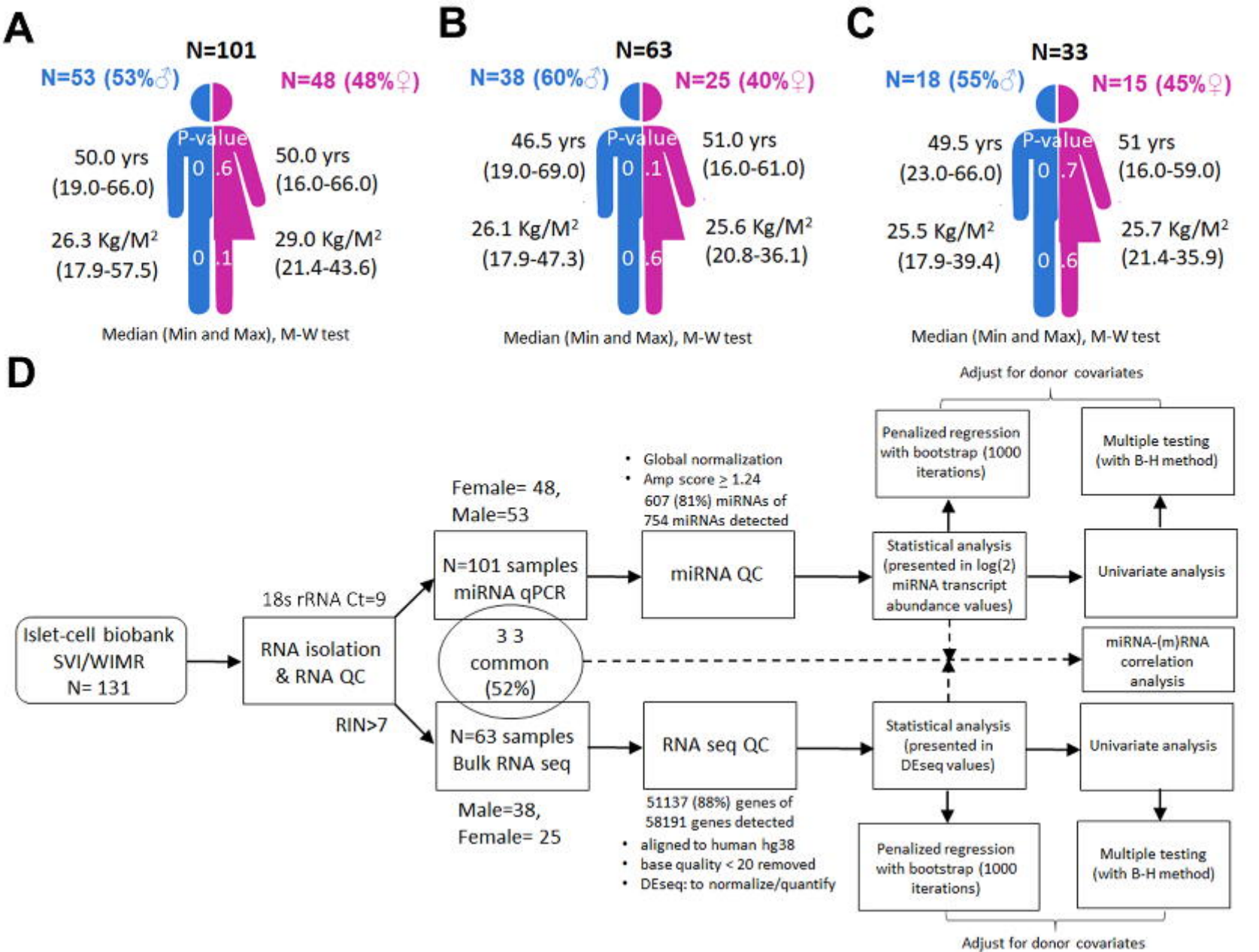
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Median (Min and Max), M-W test
D
Adjust for donor covariates
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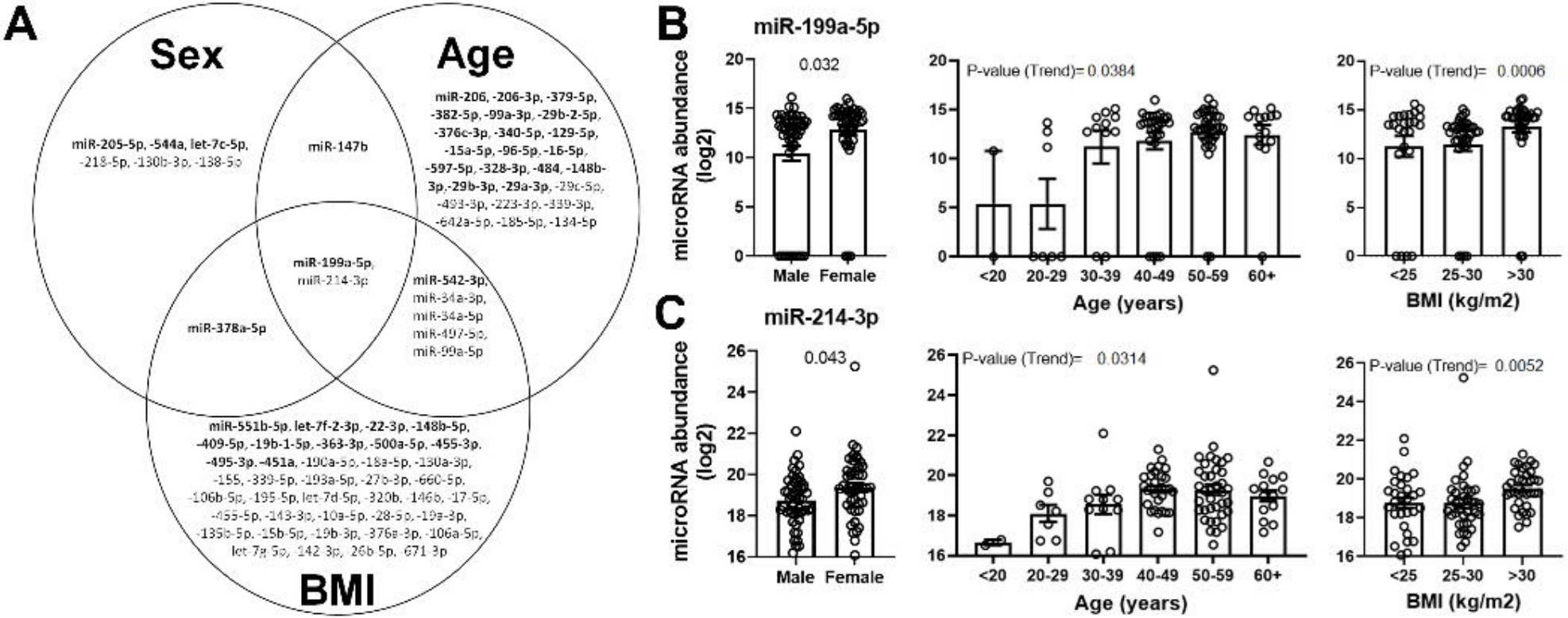
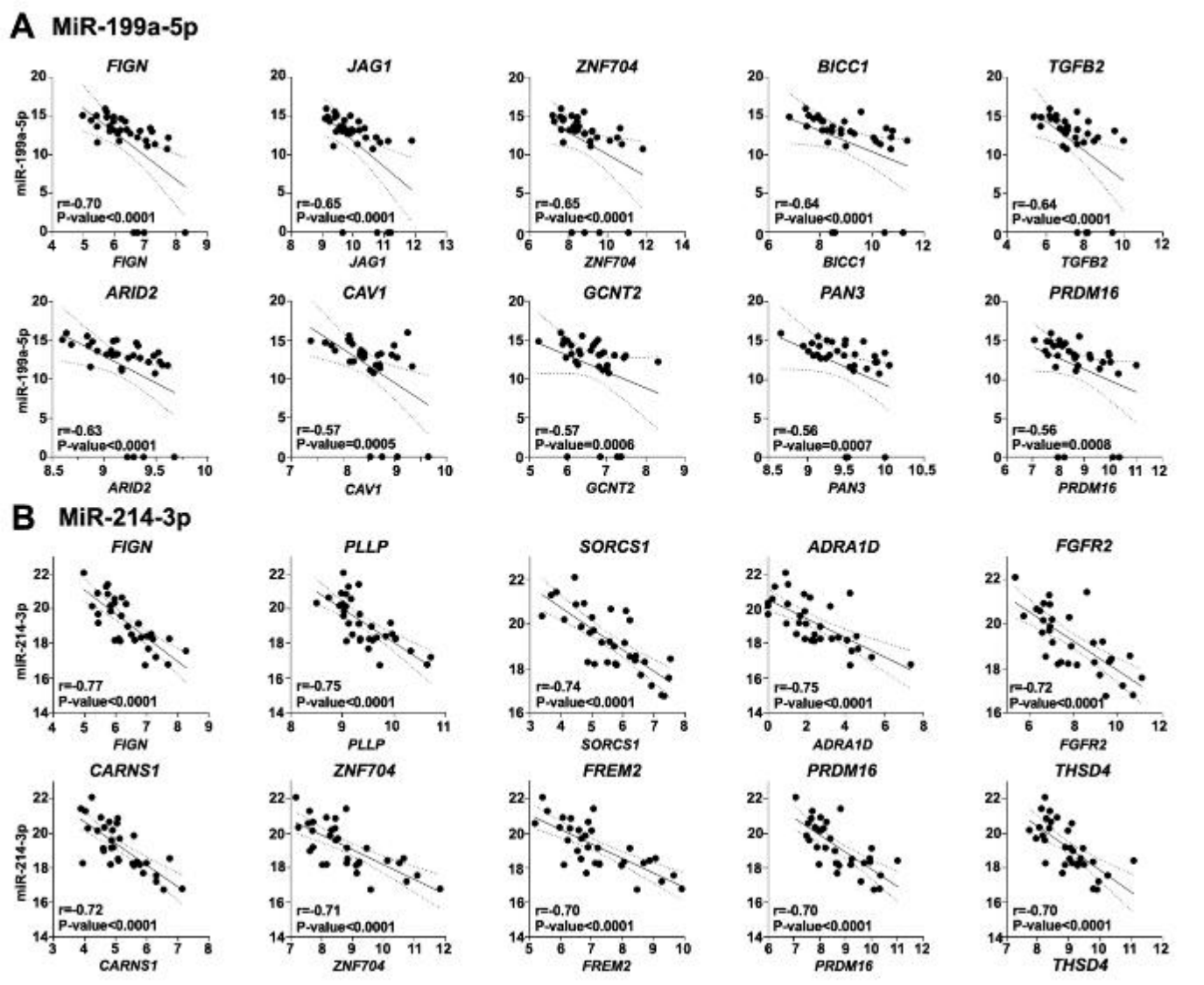
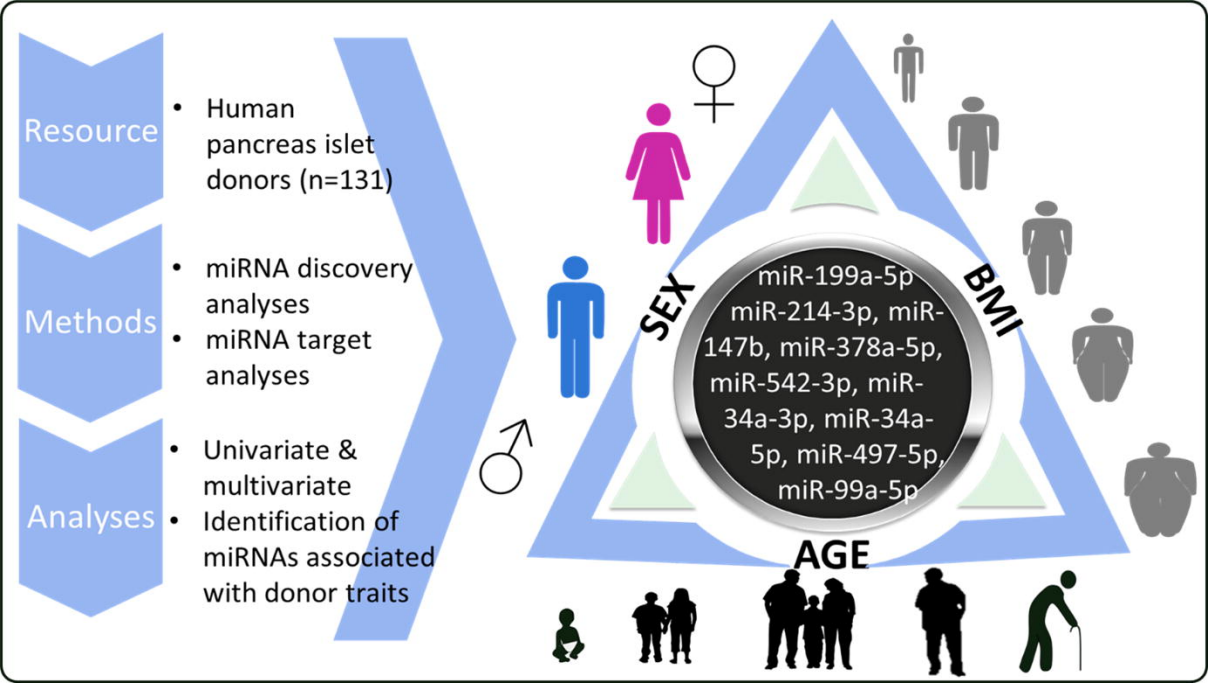
N=101 samples miRNA qPCR
miRNA QC
Statistical analysis (presented in log(2) miRNA transcript abundance values)
Univariate analysis
18s rRNA Ct=9
Islet-cell biobank SVI/WIMR N= 131
RNA isolation & RNA QC
3 3 common (52%)
miRNA-(m)RNA correlation analysis
RIN>7
N=63 samples Bulk RNA seq
RNA seq QC
Statistical analysis (presented in DEseq values)
Univariate analysis
Male=38, Female= 25
51137 (88%) genes of 58191 genes detected
aligned to human hg38
base quality < 20 removed
DEseq: to normalize/quantify
Penalized regression with bootstrap (1000 iterations)
Multiple testing (with B-H method)
Adjust for donor covariates


A
Sex
Age
BMI
miR-205-5p, -544a, let-7c-5p, -218-5p, -130b-3p, -138-5p
miR-147b
miR-206, -206-3p, -379-5p, -382-5p, -99a-3p, -29b-2-5p, -376c-3p, -340-5p, -129-5p, -15a-5p, -96-5p, -16-5p, -597-5p, -328-3p, -484, -148b-3p, -29b-3p, -29a-3p, -29c-5p, -493-3p, -223-3p, -339-3p, -642a-5p, -185-5p, -134-5p
miR-199a-5p, miR-214-3p
miR-378a-5p
miR-542-3p, miR-34a-3p, miR-34a-5p, miR-497-5p, miR-99a-5p
miR-551b-5p, let-7f-2-3p, -22-3p, -148b-5p, -409-5p, -19b-1-5p, -363-3p, -500a-5p, -455-3p, -495-3p, -451a, -190a-5p, -18a-5p, -130a-3p, -155, -339-5p, -193a-5p, -27b-3p, -660-5p, -106b-5p, -195-5p, let-7d-5p, -320b, -146b, -17-5p, -455-5p, -143-3p, -10a-5p, -28-5p, -19a-3p, -135b-5p, -15b-5p, -19b-3p, -376a-3p, -106a-5p, let-7g-5p, -142-3p, -26b-5p, -671-3p
B
miR-199a-5p
microRNA abundance (log2)
0.032
Male
Female
P-value (Trend)= 0.0384
<20
20-29
30-39
40-49
50-59
60+
Age (years)
P-value (Trend)= 0.0006
<25
25-30
>30
BMI (kg/m2)
C
miR-214-3p
microRNA abundance (log2)
0.043
Male
Female
P-value (Trend)= 0.0314
<20
20-29
30-39
40-49
50-59
60+
Age (years)
P-value (Trend)= 0.0052
<25
25-30
>30
BMI (kg/m2)


A MiR-199a-5p
FIGN
r=-0.70
P-value<0.0001
JAG1
r=-0.65
P-value<0.0001
ZNF704
r=-0.65
P-value<0.0001
BICC1
r=-0.64
P-value<0.0001
TGFB2
r=-0.64
P-value<0.0001
ARID2
r=-0.63
P-value<0.0001
CAV1
r=-0.57
P-value=0.0005
GCNT2
r=-0.57
P-value=0.0006
PAN3
r=-0.56
P-value=0.0007
PRDM16
r=-0.56
P-value=0.0008
miR-199a-5p
B MiR-214-3p
FIGN
r=-0.77
P-value<0.0001
PLLP
r=-0.75
P-value<0.0001
SORCS1
r=-0.74
P-value<0.0001
ADRA1D
r=-0.75
P-value<0.0001
FGFR2
r=-0.72
P-value<0.0001
CARNS1
r=-0.72
P-value<0.0001
ZNF704
r=-0.71
P-value<0.0001
FREM2
r=-0.70
P-value<0.0001
PRDM16
r=-0.70
P-value<0.0001
THSD4
r=-0.70
P-value<0.0001
miR-214-3p


Resource
• Human pancreas islet donors (n=131)
Methods
• miRNA discovery analyses
• miRNA target analyses
Analyses
• Univariate & multivariate
• Identification of miRNAs associated with donor traits
SEX
BMI
AGE
miR-199a-5p
miR-214-3p, miR-147b, miR-378a-5p, miR-542-3p, miR-34a-3p, miR-34a-5p, miR-497-5p, miR-99a-5p