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RESEARCH ARTICLE

Blood pathway analyses reveal differences between prediabetic subjects with or without dyslipidaemia. The Cardiovascular Risk in Young Finns Study

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Abstract

Background: Prediabetes often occurs together with dyslipidaemia, which is paradoxically treated with statins predisposing to type 2 diabetes mellitus. We examined peripheral blood pathway profiles in prediabetic subjects with (PR_D) and without dyslipidaemia (PR_0) and compared these to nonprediabetic controls without dyslipidaemia (C_0).

Methods: The participants were from the Cardiovascular Risk in Young Finns Study, including 1240 subjects aged 34 to 49 years. Genome-wide expression data of peripheral blood and gene set enrichment analysis were used to investigate the differentially expressed genes and enriched pathways between different subtypes of prediabetes.

Results: Pathways for cholesterol synthesis, interleukin-12-mediated signalling events, and downstream signalling in naïve CD8+ T-cells were upregulated in the PR_0 group in comparison with controls (C_0). The upregulation of these pathways was independent of waist circumference, blood pressure, smoking status, and insulin. Adjustment for CRP left the CD8+ T-cell signalling and interleukin-12-mediated signalling event pathway upregulated. The cholesterol synthesis pathway was also upregulated when all prediabetic subjects (PR_0 and

Abbreviations: BH-FDR, Benjamini-Hochberg false discovery rate; BP, blood pressure; C_0, normoglycaemic and normolipidaemic subjects; C_D, normoglycaemic and dyslipidaemic subjects; CV, cardiovascular; FDR, false discovery rate; FWER, family-wise error rate; GSEA, gene set enrichment analysis; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; KEGG, Kyoto Encyclopedia of Genes and Genomes; NES, normalized enrichment score; PC, principal component; PR_0, prediabetic and normolipidaemic subjects; PR_D, prediabetic and dyslipidaemic subjects; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides; YFS, Young Finns Study

PR_D) were compared with the nonprediabetic control group. No pathways were upregulated or downregulated when the PR_D group was compared with the C_0 group. Five genes in the PR_0 group and 1 in the PR_D group were significantly differentially expressed in comparison with the C_0 group.

Conclusions: Blood cell gene expression profiles differ significantly between prediabetic subjects with and without dyslipidaemia. Whether this classification may be used in detection of prediabetic individuals at a high risk of cardiovascular complications remains to be examined.

KEYWORDS

dyslipidaemia, gene expression, gene set enrichment analysis, prediabetes

1 | INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) and the subsequent development of its cardiovascular complications (CV) are increasing worldwide. A potential risk factor for the development of T2DM is prediabetes (PR), which may be defined as an intermediate state between normal glucose metabolism and T2DM. The American Diabetes Association defines PR as impaired glucose tolerance (IGT), impaired fasting glucose (IFG), or elevated HbA_{1c}.[1] Deficiency in the insulin secretion of pancreatic beta cells has a more pronounced role in PR than insulin resistance,[2] but most persons with PR are also insulin-resistant.[3]

Dyslipidaemia, which is defined by elevated total cholesterol (TC), low density lipoprotein (LDL) cholesterol (LDL-C), nonhigh density lipoprotein cholesterol (non-HDL-C) or triglycerides (TGs), or low HDL-C,[4] commonly occurs together with T2DM. A typical pattern of lipid abnormalities in diabetic patients includes hypertriglyceridaemia, low HDL-C, and a presence of small dense LDL-C particles.[5] The same pattern, known as atherogenic dyslipidaemia, has been shown to occur already during the prediabetic stage.[6,7] Metabolically, low absorption efficiency and high synthesis of cholesterol are also related to an elevated serum glucose level and insulin resistance.[8,9] However, whether the progression or development of CV complications will differ in prediabetic patients with and without dyslipidaemia is not well known.

In atherogenic dyslipidaemia, statins are widely used as first-line drugs. Because the treatment goal for serum LDL-C is more stringent in patients with T2DM than in those without T2DM, high-dose statin treatment may be needed.[4] The use of statins in prediabetic subjects may be complicated because these drugs can increase the risk of incident T2DM,[10] and by affecting insulin sensitivity, insulin secretion and glucose transport increase plasma glucose levels.[11] Despite this controversy, statins yield an overall benefit in preventing vascular events, and therefore, these drugs are widely recommended in current treatment guidelines also for the treatment of dyslipidaemia in T2DM patients.[4] With this clinical background, it is rational to seek better understanding of the metabolic differences of PR subphenotypes with and without dyslipidaemia.

Gene expression and pathway profiling of blood and tissue samples may provide better understanding of the pathogenesis of PR and its association to dyslipidaemia. An earlier metabolomic investigation of plasma from nondiabetic subjects with reduced insulin sensitivity showed alterations in lipid metabolic pathways, steroid hormone biosynthesis, and bile acid metabolism.[12] Most of these pathways and certain amino acid metabolism pathways have also been found to be differentially regulated in the liver of pigs with impaired incretin (a group of metabolic hormones that stimulate a decrease in blood glucose levels) function.[13] Altered pathways in lipid metabolism, insulin action, inflammatory response, and complex oxidative processes have also been revealed from subcutaneous adipose and muscle tissue from nondiabetic, insulin-resistant subjects.[14,15] However, these earlier metabolic studies did not take into account whether plasma lipid abnormalities were present in the subjects.

It has been suggested that insulin resistance elicits dyslipidaemia either mechanically or by means of genetic linkage, but further validation is still needed.[2,3] We aimed to identify the metabolic pathways and gene expression associated with the prediabetic state, with special respect to a division based on the subjects' dyslipidaemia status. To pinpoint dysregulated metabolic pathways associated with these PR subphenotypes, we performed a gene set enrichment analysis (GSEA) and also otherwise analysed gene-wise differences between PR subphenotypes and controls.

2 | MATERIALS AND METHODS

2.1 | Population

The Cardiovascular Risk in Young Finns Study (YFS) is a Finnish longitudinal population study on the evolution of CV risk factors from childhood to adulthood; the sample and methods have been described in detail elsewhere.[16,17] The present study included 1240 subjects who were not diagnosed with T1DM or T2DM (in 2012), were not on medication for hypertension or hypercholesterolaemia, and for whom complete gene expression data as well as data on lipids, glucose, and clinical characteristics were available.

The study plan was approved by the ethics committees of all participating universities, and the study protocol of each study phase corresponded with the proposal by the World Health Organization. All subjects gave written informed consent, and the study was conducted in accordance with the Declaration of Helsinki.

2.2 | Clinical and biochemical measurements

Height and weight were measured, and body mass index (BMI) was calculated as weight in kilograms divided by height in metres squared. Waist circumference was measured by using an anthropometric tape at the midpoint between the iliac crest and the lowest rib to the nearest 0.1 cm. Blood pressure was measured 3 times after a 5 minute rest with a random zero sphygmomanometer and was estimated as the average of the 3 measurements.

Venous blood samples were drawn after an overnight fast for the determination of serum lipid levels, glucose, insulin, glycated haemoglobin A1c (HbA1c), and high-sensitive C-reactive protein (hs-CRP). Standard enzymatic methods were used for serum TC, TG, and HDL-C determinations. Low density lipoprotein cholesterol was calculated by the Friedewald formula in participants with TG levels less than 4.0 mmol/L.[18] Nonhigh density lipoprotein cholesterol was calculated as TC-HDL-C. Glucose concentrations were determined by the enzymatic hexokinase method. Serum insulin was measured with immunoassay and HbA1c with an immunoturbidimetric method. High-sensitive C-reactive protein was determined immunoturbidimetrically. Details of all of the methods have been previously described elsewhere.[19]

2.3 | Ribonucleic acid isolation, microarrays, and data processing

Ribonucleic acid was isolated, and the gene expression levels were analysed by using commercially available kits. Expression data were analysed in R (http://www.r-project.org/) by using the Bioconductor packages (http://www.bioconductor.org/). Details of the process have been previously described elsewhere.[20]

2.4 | Definition of prediabetes and dyslipidaemia

The classification of PR was based on fasting plasma glucose and HbA1c according to the criteria of the American Diabetes Association.[1] People with impaired IFG, ie, PR, were defined as having a fasting plasma glucose level of 5.6 to 6.9 mmol/L or HbA1c of 5.7 to 6.4% (38-46 mmol/L) and not diagnosed with T2DM. The diagnosis of T2DM included subjects with a fasting plasma glucose level of over 7.0 mmol/L or HbA1c of over 6.5% (48 mmol/L) or those with reported use of oral glucose-lowering medication or insulin (but had not reported having T1DM) or who had a reported diagnosis of T2DM by a physician.

Dyslipidaemia was defined according to the European guidelines.[4] The criteria for dyslipidaemia were TC greater than 5.0 mmol/L, LDL-C greater than 3.0 mmol/L, HDL-C less than 1.0 mmol/L in men and less than 1.2 mmol/L in women, non-HDL-C greater than 3.8 mmol/L, or TG greater than 1.7 mmol/L.

2.5 | Statistical analysis

Gene sets were collected from 5 publicly available collections: BioCarta (http://cgap.nci.nih.gov/Pathways/BioCarta_Pathways), KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/), Reactome (http://www.reactome.org/), National Cancer Institute Pathway Interaction Database (http://pid.nci.nih.gov/), and HumanCyc (http://humancyc.org/). Enrichment analysis was performed by using each gene set separately. To avoid too narrowly or too broadly defined functional gene sets, pathways containing less than 10 or more than 200 genes were excluded. As a result, 1078 pathways were included in the study. The reduced number of pathways potentially increases the power of the analysis by decreasing the multiple testing correction burden.

The study population was divided into 4 subphenotypes as follows: prediabetic individuals with (PR_D) or without dyslipidaemia (PR_0) and normoglycaemic (nonprediabetic) control (C) subjects with (C_D) or without (C_0) dyslipidaemia. All prediabetic subjects (PRs), regardless of dyslipidaemia status were compared with the nonprediabetic control group (C_0 and C_D together). The PR_0 and PR_D groups were individually compared with the C_0 group and also to each other (PR_0 vs PR_D). We also did similar analyses and examined whether the results differ when the dyslipidaemia status definition was based on the high LDL-C level (LDL > 3.0 mmol/L) only. The baseline characteristics of the groups were compared by using the *t* test for continuous variables and a χ^2 test for proportions.

Potential population stratification was taken into account by using principal components (PCs) computed from all genotypes as covariates.[21] Based on a scree plot, the 7 first PCs were used. In addition to the PCs, the analyses were adjusted by age, sex, BMI or waist circumference, smoking, insulin, systolic and diastolic blood pressure, and hs-CRP. R language was used for adjusting the gene expression data. After the adjustment, GSEA software (http://www.broad.mit.edu/gsea)[22,23] was used to analyse the association of gene pathways with the phenotype. The pathways were considered to be significantly upregulated or downregulated when the false discovery rate (FDR) was smaller than 0.10 and the family-wise error rate (FWER) was smaller than 0.05 after 1000 permutation cycles. False discovery rate less than 0.25 can be considered significant according to the criteria recommended by Subramanian et al.[22]

The expression of individual genes in the same setting was analysed by using the phenoTest R package with a Benjamini-Hochberg-FDR-corrected *P* value of less than or equal to .05 and log2 fold change of greater than or equal to 1.5 as the significance level. The analysis was adjusted with age, sex, BMI, and the first 7 PCs. For box plots, the statistical significance of the difference in gene expression was assessed by using the nonparametric Wilcoxon signed-rank test.

3 | RESULTS

The demographics of the study population, when the division is based on any type of dyslipidaemia, or hypercholesterolaemia defined as a high LDL-C (LDL > 3.0 mmol/L) only, are presented in Table 1. Of the nonmedicated subjects with PR, 79.5% had dyslipidaemia and 66.0% hypercholesterolaemia defined as LDL greater than 3.0 mmol/L. When comparing all PR individuals to the nonprediabetic control group (C), GSEA identified upregulation of cholesterol biosynthesis pathways in all of the used but differently adjusted models 1 to 3 (FDR < 0.014 for all) (Table 2). A positive normalized enrichment score (NES) indicated that all the pathways were upregulated. The leading-edge subsets containing the most upregulated genes are almost identical in

the KEGG steroid and HumanCyc cholesterol biosynthesis pathways (Table SS1). In the PR versus C group comparison, superpathway of methionine degradation remained significantly enriched in models 1 and 2 (FDR < 0.006 and <0.015, respectively). However, in model 3, the additional adjustment with hs-CRP abolished the association.

Two additional pathways, cholesterol biosynthesis II (via 24,25-dihydrolanosterol) and cholesterol biosynthesis III (via desmosterol) from HumanCyc, were closely and significantly co-enriched with the cholesterol biosynthesis pathway because they all share the same genes. Hence, they are not shown in the tables.

These KEGG and HumanCyc pathways were also upregulated when the PR_0 group was compared with the corresponding control group without PR and dyslipidaemia (C_0) after adjustment for age, sex, BMI, and the first 7 PCs (Table 3). In addition, in this setting, the pathways for interleukin-12 (IL12)-mediated signalling events and downstream signalling in naive CD8+ T-cells were also significantly

TABLE 1 Demographics of the study population according to prediabetes/control (PR/C), dyslipidaemia (D/0), and hypercholesterolaemia subtype status (HC/NC)

	C_0	PR_0	C_D	PR_D
Number of subjects	256	79	598	307
Age (years)	40.1 (4.87)	41.2 (5.00)	41.4 (5.07)[a]	42.4 (4.97)[a]
Male (%)	26.2	41.8[a]	42.0[a]	63.8[a]
Total cholesterol (mmol/L)	4.36 (0.40)	4.33 (0.51)	5.42 (0.89)[a]	5.59 (0.89)[a]
LDL cholesterol (mmol/L)	2.51 (0.35)	2.53 (0.43)	3.53 (0.76)[a]	3.63 (0.79)[a]
HDL cholesterol (mmol/L)	1.49 (0.25)	1.40 (0.27)[a]	1.34 (0.35)[a]	1.25 (0.32)[a]
Non-HDL cholesterol (mmol/L)	2.88 (0.36)	2.92 (0.49)	4.09 (0.83)[a]	4.33 (0.86)[a]
Triglycerides (mmol/L)	0.82 (0.28)	0.88 (0.29)	1.25 (0.73)[a]	1.68 (1.55)[a]
Systolic BP (mmHg)	113 (12.3)	117 (12.0)[a]	118 (13.6)[a]	123 (14.0)[a]
Diastolic BP (mmHg)	70.3 (8.90)	73.9 (9.78)[a]	73.7 (10.3)[a]	78.5 (10.4)[a]
hs-C-reactive protein (mg/L)	1.09 (1.78)	2.23 (4.47)[a]	1.37 (2.03)[a]	1.77 (2.26)[a]
Glucose (mmol/L)	4.99 (0.34)	5.64 (0.47)[a]	5.08 (0.33)[a]	5.71 (0.41)[a]
HbA1c (%)	5.29 (0.18)	5.58 (0.28)[a]	5.35 (0.17)[a]	5.63 (0.26)[a]
HbA1c (mmol/L)	34.4 (2.00)	37.4 (2.95)[a]	35.0 (1.92)[a]	38.0 (2.76)[a]
Insulin (mU/L)	5.73 (3.40)	8.68 (6.04)[a]	7.59 (5.05)[a]	11.0 (7.43)[a]
Body mass index (kg/m^2)	23.6 (3.39)	26.2 (4.90)[a]	25.6 (3.99)[a]	28.3 (4.75)[a]
Waist circumference (cm)	82.5 (10.2)	90.4 (14.3)[a]	88.9 (11.9)[a]	97.7 (12.8)[a]
Daily smokers (%)	7.81	17.7[a]	13.2[a]	16.2[a]
	C_{NC}	PR_{NC}	C_{HC}	PR_{HC}
Number of subjects	362	127	480	247
Age (years)	39.9 (4.79)	40.9 (4.87)	41.8 (5.07)[b]	42.8 (4.99)[b]
Male (%)	28.7	48.0[b]	42.5[b]	63.6[b]
Total cholesterol (mmol/L)	4.36 (0.51)	4.39 (0.53)	5.64 (0.73)[b]	5.75 (0.78)[b]
LDL cholesterol (mmol/L)	2.52 (0.35)	2.53 (0.38)	3.75 (0.64)[b]	3.84 (0.67)[b]
HDL cholesterol (mmol/L)	1.41 (0.34)	1.29 (0.34)[b]	1.38 (0.32)	1.29 (0.29)[b]
Non-HDL cholesterol (mmol/L)	2.95 (0.42)	3.10 (0.52)[b]	4.27 (0.72)[b]	4.47 (0.78)[b]
Triglycerides (mmol/L)	0.97 (0.50)	1.27 (0.77)[b]	1.15 (0.54)[b]	1.39 (0.62)[b]
Systolic BP (mmHg)	114 (12.2)	119 (12.3)[b]	118 (13.9)[b]	123 (14.4)[b]
Diastolic BP (mmHg)	71.3 (9.61)	75.3 (10.0)[b]	73.6 (10.2)[b]	78.5 (10.6)[b]
hs-C-reactive protein (mg/L)	1.39 (2.27)	2.06 (3.74)	1.19 (1.72)	1.80 (2.34)[b]
Glucose (mmol/L)	5.01 (0.35)	5.70 (0.48)[b]	5.08 (0.32)[b]	5.69 (0.40)[b]
HbA1c (%)	5.31 (0.18)	5.58 (0.26)[b]	5.36 (0.18)[b]	5.64 (0.25)[b]
HbA1c (mmol/L)	34.5 (1.95)	37.4 (2.86)[b]	35.1 (1.95)[b]	38.2 (2.69)[b]
Insulin (mU/L)	6.50 (4.42)	10.3 (7.48)[b]	7.21 (4.49)[b]	9.99 (6.13)[b]
Body mass index (kg/m^2)	24.1 (3.87)	27.3 (5.23)[b]	25.6 (3.85)[b]	28.1 (4.69)[b]
Waist circumference (cm)	84.1 (10.9)	94.1 (14.8)[b]	88.9 (11.9)[b]	96.8 (12.6)[b]
Daily smokers (%)	11.3	20.5[b]	11.4	14.6

Definitions/abbreviations: PR_0 indicates prediabetes without dyslipidaemia; PR_D, prediabetes with dyslipidaemia; C_D, nonprediabetic subjects with dyslipidaemia; C_0, healthy subjects without prediabetes or dyslipidaemia; PR_{NC}, prediabetes without hyper-LDL cholesterolaemia; PR_{HC}, prediabetes with hyper-LDL cholesterolaemia; C_{HC}, nonprediabetic subjects with hyper-LDL cholesterolaemia; C_{NC}, healthy subjects without prediabetes or hyper-LDL cholesterolaemia; hs, high sensitive.Statistics: *t* test or χ^2 test when appropriate. Values are mean (±SD) or proportions.

[a]Difference as compared with C_0, $P < .05$.

[b]Difference as compared with C_{NC}, $P < .05$.

TABLE 2 Pathways enriched in all prediabetic subjects (PRs) in comparison with all control subjects without prediabetes (C). All pathways were upregulated as indicated by a positive NES

	NES	Enrichment *P* Value	FDR	FWER
Model 1				
Steroid biosynthesis[a]	2.13	<.001	0.008	0.006
Cholesterol biosynthesis[b]	2.04	<.001	0.007	0.009
Superpathway of cholesterol biosynthesis[b]	1.91	<.001	0.009	0.034
Superpathway of methionine degradation[b]	1.97	.002	0.006	0.019
Model 2				
Steroid biosynthesis[a]	2.06	.002	0.010	0.010
Cholesterol biosynthesis[b]	1.99	<.001	0.005	0.007
Superpathway of methionine degradation[b]	1.97	.002	0.015	0.045
Model 3				
Steroid biosynthesis[a]	2.06	<.001	0.014	0.012
Cholesterol biosynthesis[b]	2.00	<.001	0.003	0.006

Statistics: Model 1: Gene set enrichment analysis adjusted for age, sex, BMI, and the first 7 PCs; Model 2: Model 1 + additionally adjusted for waist circumference (instead of BMI), systolic and diastolic BP, smoking, and insulin; Model 3: Model 2 + additionally adjusted for hs-CRP.

[a]Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

[b]HumanCyc pathways. Abbreviations: FDR indicates false discovery rate; FWER, family-wise error rate; NES, normalized enrichment score; PC, principal component; hs, high sensitive.

upregulated. When further adjusted for waist circumference (instead of BMI), blood pressure, smoking, and insulin, all other pathways except the HumanCyc superpathway of cholesterol synthesis remained significant. In the fully adjusted model 3, the association of steroid and cholesterol biosynthesis pathways was abolished after additional adjustment for hs-CRP, leaving only the downstream signalling in naive CD8+ T-cells and IL12-mediated signalling events significantly upregulated.

When the PR_D group was compared with the C_0 group, no pathways were significantly enriched in any of the models. In the PR_0 versus PR_D group comparison one Reactome pathway, Cytochrome P450 arranged by substrate type was upregulated in the PR_D group (NES 2.09, $P < .001$, FDR 0.041, and FWER 0.026) after adjustment for age, sex, BMI, and the 7 first PCs. Further adjustment in models 2 and 3, similar as in other analyses, abolished the association.

When comparing prediabetic subjects with normal LDL-C (<3.0 mmol/L) (PR_{NC}) to normoglycaemic subjects with normal LDL-C (C_{NC}), 1 pathway for cholesterol biosynthesis remained significantly upregulated in all models 1 to 3 (Table 4). In prediabetic subjects with high LDL-C (≥3.0 mmol/L) (PR_{HC}), no pathways were significantly

TABLE 3 Pathways enriched in prediabetic subjects without dyslipidaemia (PR_0) in comparison with control subjects without prediabetes and dyslipidaemia (C_0). All pathways were upregulated as indicated by a positive NES

	NES	Enrichment *P* Value	FDR	FWER
Model 1				
Steroid biosynthesis[a]	2.04	<.001	0.020	0.022
Cholesterol biosynthesis[b]	2.10	<.001	0.001	0.001
Superpathway of cholesterol biosynthesis[b]	1.94	.002	0.007	0.015
Downstream signalling in naïve CD8+ T-cells[c]	1.99	<.001	0.020	0.038
IL12-mediated signalling events[c]	2.00	.002	0.035	0.036
Model 2				
Steroid biosynthesis[a]	1.96	<.001	0.048	0.044
Cholesterol biosynthesis[b]	1.97	<.001	0.009	0.015
Downstream signalling in naïve CD8+ T-cells[c]	2.09	.002	0.006	0.007
IL12-mediated signalling events[c]	1.99	.002	0.014	0.030
Model 3				
Downstream signalling in naïve CD8+ T-cells[c]	2.08	<.001	0.016	0.012
IL12-mediated signalling events[c]	2.06	<.001	0.011	0.022

Statistics: Model 1: Gene set enrichment analysis adjusted for age, sex, BMI, and the first 7 PCs; Model 2: Model 1 + additionally adjusted for waist circumference (instead of BMI), systolic and diastolic BP, smoking, and insulin; Model 3: Model 2 + additionally adjusted for hs-CRP.

[a]Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

[b]HumanCyc pathways.

[c]National Cancer Institute Pathway Interaction Database(NCI PID) pathways. Abbreviations: FDR indicates false discovery rate; FWER, family-wise error rate; NES, normalized enrichment score; PC, principal component; hs, high sensitive.

enriched as compared with the C_{NC} group. When comparing the 2 prediabetic groups to each other (PR_{NC} vs PR_{HC}), 1 pathway from National Cancer Institute Pathway Interaction Database was significantly upregulated. Regulation of cytoplasmic and nuclear SMAD2/3 signalling was enriched in the PR_{HC} group in models 1 (NES 2.03, *P* .002, FDR 0.029, and FWER 0.022) and 2 (NES 2.00, *P* < .001, FDR 0.042, and FWER 0.033) but not in model 3.

The expression of individual pathway genes was not statistically significant in any PR phenotype when compared with the C_0 group. This is explained by the fact that GSEA considers all expressed genes by rank without a fold-change threshold. Therefore, we also tested gene-wise differences between PR subphenotype groups with a less stringent cut-off value for log2 fold change (>1.2) and a Benjamini-Hochberg-FDR-corrected *P* value of less than or equal to .05 (Table S2). In gene-wise analysis, we identified 5 genes upregulated in the PR_0 group as compared with the C_0 group, including type 1 neurotrophic tyrosine kinase receptor; granzyme B (*GZMB*); perforin 1 (*PRF1*); killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail, 4 (*KIR2DL4*); and family with sequence similarity 179 member A. One gene, secretory leukocyte peptidase inhibitor (*SLPI*), was upregulated in the PR_D subjects as compared with the C_0 group. The trend analyses for these 6 genes are shown in Figure 1. In PR versus C, PR_0 versus PR_D, and PR_{NC} versus PR_{HC} group comparisons, no genes were differentially expressed.

4 | DISCUSSION

Our analysis of peripheral blood cell mRNA expression shows, for the first time, that the pathway profiles differ significantly between prediabetic subphenotypes with and without dyslipidaemia. We observed that, compared with normoglycaemic and normolipidaemic controls, the cholesterol biosynthesis pathway was upregulated in normolipidaemic prediabetic individuals but not in those with both PR and dyslipidaemia. Also, pathways related to the immune response were upregulated only in the PR_0 group. It is not surprising that pathway analysis identified differences between prediabetic subphenotypes. However, using the most recent pathway databases, our analysis pinpointed the specific pathways that were upregulated.

The enrichment of the cholesterol biosynthesis pathway was independent of both BMI and waist circumference. Parallel results have been reported by Gylling et al,[9] who assayed cholesterol precursors and markers of cholesterol synthesis and absorption from plasma. In their study, markers of cholesterol synthesis were already increased in subjects with IFG and cholesterol metabolism was regulated more by peripheral insulin sensitivity than obesity. In the present study, the enrichment remained significant until the analysis was adjusted for hs-CRP. This suggests that an upregulated cholesterol biosynthesis pathway is related to the increased overall inflammation as measured by hs-CRP. Interestingly, serum hs-CRP concentration has been previously associated with dietary cholesterol absorption but not synthesis of cholesterol in subjects with IFG or IGT and features of the metabolic syndrome cholesterol metabolism.[24]

The mechanism through which PR in the absence of dyslipidaemia regulates cholesterol metabolism gene pathways in blood cells remains open. A possible reason for the upregulation of cholesterol synthesis in the PR_0 group is the cholesterol deprivation inside the blood cells due to the lack of extra cholesterol available in the plasma. In leukocytes, the expression of certain genes that are included in the KEGG steroid biosynthesis pathway has been found to be associated with plasma lipid levels. The expression is hypothesized to be activated by peroxisome proliferative activated receptors.[25] However, we did not observe changes in the expression of peroxisome proliferative activated receptors. Also, we did not observe gene-wise changes in the expression of HMG-CoA reductase (*HMGCR*), the rate-limiting step in cholesterol metabolism, or sterol-regulatory element-binding protein, which regulates the transcription of *HMGCR*. The upregulated pathways consist of the latter half of the cholesterol biosynthesis pathway, with farnesyl pyrophosphate being the first intermediate. The first sterol intermediate is lanosterol, and the subsequent reactions define the postsqualene part of the pathway. In this portion of the pathway, the demethylation of lanosterol has been suggested to act as the rate-limiting step.[26]

Because isolated IFG and IGT are characterized by different patterns of lipid changes,[27] they presumably affect the lipid metabolism by distinct mechanisms. The downregulation of the cholesterol biosynthesis pathway has been previously associated in insulin resistance in adipose tissue,[14] but in our study, the same pathway was upregulated in peripheral blood. Analogous results have been published related to the mitogen-activated protein kinase signalling pathway in insulin resistance in metabolic syndrome—the pathway is upregulated in muscle tissue but downregulated in blood.[28,29] On the other hand,

TABLE 4 Pathways enriched in subjects who had prediabetes but no hyper-LDL cholesterolaemia (PR_{NC}) in comparison with those without prediabetes and hyper-LDL cholesterolaemia (C_{NC}) (LDL ≤ 3.0 mmol/L). All pathways were upregulated as indicated by a positive NES

	NES	Enrichment *P* Value	FDR	FWER
Model 1				
Superpathway of cholesterol biosynthesis[a]	1.92	.002	0.052	0.030
Model 2				
Superpathway of cholesterol biosynthesis[a]	1.87	.002	0.054	0.042
Model 3				
Superpathway of cholesterol biosynthesis[a]	1.96	<.001	0.015	0.012

Statistics: Model 1: Gene set enrichment analysis adjusted for age, sex, BMI, and the first 7 PCs; Model 2: Model 1 + additionally adjusted for waist circumference (instead of BMI), systolic and diastolic BP, smoking, and insulin; Model 3: Model 2 + additionally adjusted for hs-CRP.

[a]HumanCyc pathways. Abbreviations: FDR indicates false discovery rate; FWER, family-wise error rate; NES, normalized enrichment score; PC, principal component; hs, high sensitive.

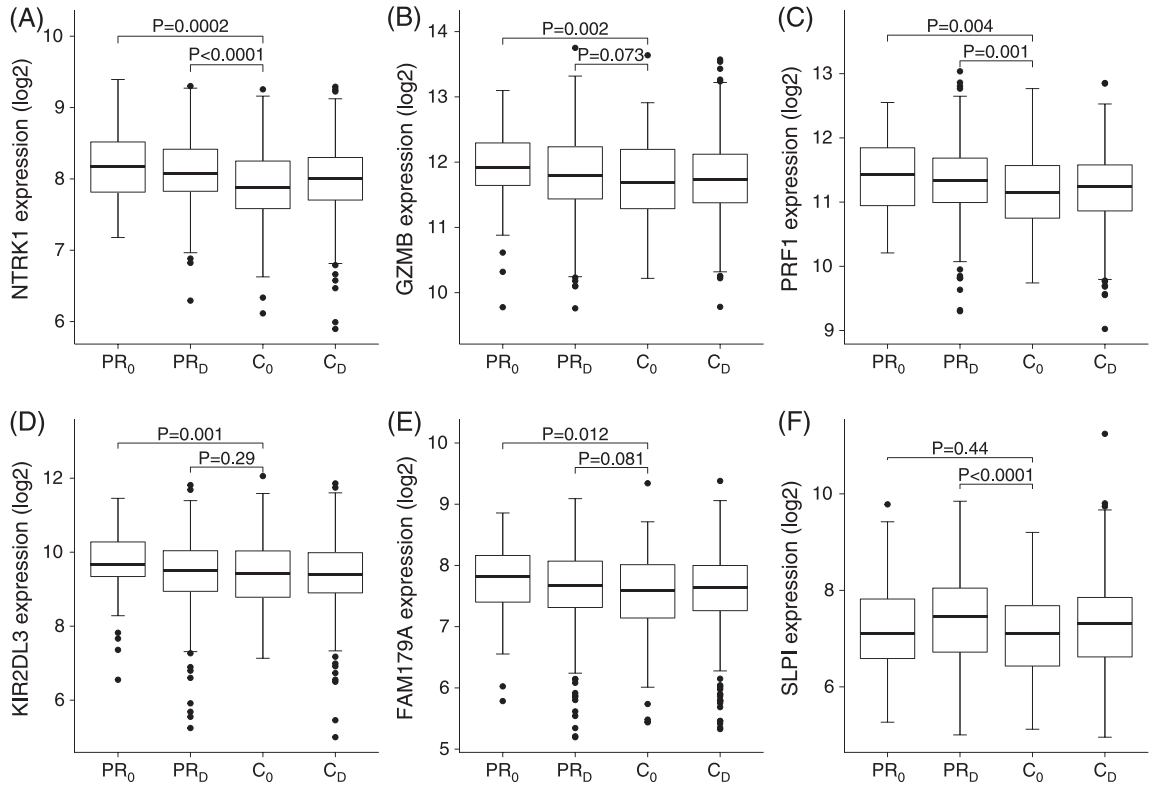

FIGURE 1 Gene expression changes of (A), type 1 neurotrophic tyrosine kinase receptor (NTRK1), (B), granzyme B (GZMB), (C), perforin 1 (PRF1), (D), KIR2DL3, (E), 179 member A (FAM179A), and (F), secretory leukocyte peptidase inhibitor (SLPI) genes over prediabetes and control phenotypes

1 study has demonstrated that the mechanisms that regulate gene expression in liver and mononuclear leukocytes are similar and that these leukocytes can be used to predict the level of expression of *HMGCR* and LDL receptor genes.[30] This could indicate that the hepatic cholesterol production is also increased in the PR_0 group, although we did not observe increased expression of these 2 genes.

Recent data suggest that nonalcoholic fatty liver disease (NAFLD) results mainly from disturbed hepatic cholesterol homeostasis and the hepatic accumulation of free cholesterol.[31] If the cholesterol synthesis pathway is upregulated in the liver, the newly synthesized cholesterol may promote the pathogenesis of NAFLD because cholesterol export and bile acid synthesis pathways were not upregulated. This hypothesis is supported by a Japanese study that showed a positive association between NAFLD and IFG, independently of T2DM risk factors.[32] Nonalcoholic fatty liver disease is also considered to be a consequence of insulin resistance,[33] but it is also an independent risk factor of T2DM, particularly in individuals with IFG.[34]

Based on above reasons, potentially increased hepatic cholesterol production would imply that the onset of PR launches a cascade leading to hypercholesterolaemia and/or NAFLD, which highlights the importance of early detection of PR and prevention of T2DM through lifestyle intervention. Guidelines in condensed form have been provided for building up an effective intervention programme; the IMAGE toolkit[35] also gives instructions for evaluation and quality assurance. In addition to working at the patient level, actions at policy and environmental levels are needed for sustainable diabetes prevention.[36]

The enrichment of the IL12 signalling pathway remained significant in all models when the PR_0 group was compared with the C_0 group. Elevated IL12 levels have been previously shown to be dependent on hs-CRP[37] and peripheral insulin resistance[38] in T2DM. Because the IL12 pathway remained upregulated when the analysis was adjusted with both serum insulin level and hs-CRP, it may be suggested that, in the PR_0 group, the activation of the IL12 pathway is mediated by another mechanism. When only the LDL-C levels were taken into account, the pathway profiles were similar to the ones of all prediabetic subjects, ie, when the dyslipidaemia status was not considered. This suggests that the upregulation of IL12-mediated and CD8+ T-cell pathways could be partly related to hypertriglyceridaemia or low HDL-C.

The enrichment of cholesterol biosynthesis and inflammation-related pathways was seen in the PR_0 group when compared with the C_0 group but not when compared with the PR_D group, which implies that the metabolic differences between the 2 PR subphenotypes are small. However, whether this difference will evolve over time requires longitudinal studies.

The analysis of individual genes revealed only a moderate increase in gene expression. Some of the genes have been previously associated with metabolic dysfunction. Only one gene, *SLPI*, which is a potent inhibitor of the inflammatory cascade,[39] was upregulated in the PR_D group. The upregulation of *SLPI* has also been previously shown to correlate negatively with HDL-C and positively with HbA1c. This may be due to an attempt to counterbalance the low-grade inflammation associated with PR and dyslipidaemia.[40] The expression of LDL receptor or scavenger receptor genes[41] in the PR_0 or PR_D groups was not different compared with the C_0 group. Because scavenger receptors are key molecules in the formation of atherosclerotic

plaques,[42] our results imply that PR combined with dyslipidaemia does not directly cause atherosclerosis, which is also stated by Grundy.[2]

Three of 5 genes that were slightly upregulated in the PR_0 group—*GZMB*, *PRF1*, and *KIR2DL4*—have been found to be downregulated after exposure to high blood glucose in normoglycaemic controls. In T2DM patients, the expression levels of these genes have been reported to be low already and hardly affected by hyperglycaemia.[43] These genes are typically expressed in cells with cytotoxic functions, such as CD8+ T-cells.[44] Granzyme B and *PRF1* are also included in the downstream signalling in the naive CD8+ T-cell pathway, which was upregulated in the PR_0 group but not in the PR_D group in comparison with the C_0 group. The reason why *GZMB*, *PRF1*, and *KIR2DL4* were upregulated in the PR_0 group but not in the PR_D group and expressed in lower levels in T2DM patients in the study by van der Pouw Kraan et al[43] might be that 1 or more of the components of dyslipidaemia co-regulate the expression of these genes; the T2DM patients in their study[43] met the elevated TG criterion of dyslipidaemia used in our study. However, in another study, the plasma level of granzyme B correlated positively with fasting glucose and HbA1c, as well as with TGs, TC, and LDL-C.[45]

The present study has some limitations. A major one is that profiling gene expression from peripheral blood cells makes it challenging to speculate how the expression levels represent the gene expression in other tissues. Another limitation is that no glucose tolerance tests were performed on the study population and the definition of PR was based only on fasting plasma glucose and HbA1c levels. Some studies,[46,47] but not all,[48] have shown that IGT is a better predictor of CV complications than IFG. This raises the question whether there are differences in gene expression and pathway profiles when PR is diagnosed by either IFG or IGT. However, the HbA1c cut-off point for PR has a high specificity to identify cases of IGT[1] and also subsequent 6 year diabetes incidence.[49] In addition, the Finnish gene pool has been shown to be distinctive and the results may not be directly generalizable to populations with a different ethnic background. We also recognize that microarray studies are limited by multiple testing problems and false positives.

In summary, our data indicate that blood cell gene expression pathway profiles differ significantly between prediabetic subphenotypes with and without dyslipidaemia. The pathway analysis identified upregulated pathways, including cholesterol biosynthesis, IL12-mediated signalling, and signalling in naïve CD8+ T-cells in prediabetic individuals only in the absence of dyslipidaemia. However, whether this classification may be used in, eg, early-phase detection of individuals at a high risk of CV complications, should be further examined in longitudinal studies. The clinical implication is that physicians should actively screen patients for PR and dyslipidaemia and encourage especially those with PR to permanent lifestyle changes with active follow-ups.

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CONFLICTS OF INTEREST

The authors declare that there is no duality of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS

JL contributed to the study design, statistical analyses, data interpretation, and drafting of the manuscript. TT and LPL contributed to the statistical analyses and critical revision of the manuscript. IS contributed to the study design, statistical analyses, and critical revision of the manuscript. ER, NM, MW, TI, NHK, TR, and MJ contributed to the data collection and critical revision of the manuscript. JV contributed to the initial design of YFS, cohort collection, and critical revision of the manuscript. MK contributed to obtaining funding, cohort collection, and critical revision of the manuscript. OR led YFS and contributed to obtaining funding, as well as cohort collection and critical revision of the manuscript. TL supervised the research and contributed to the study design, obtaining funding, and cohort collection, in addition to reviewing and editing the manuscript. All authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

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