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ABSTRACT

Objective: Bile acids (BAs) are signaling molecules controlling lipid and glucose metabolism. Since BA alterations are associated with obesity and insulin resistance, plasma BAs have been considered candidates to predict type 2 diabetes (T2D) risk. We aimed to determine (1) the association of BAs with glucose homeostasis parameters and (2) their predictive association with the risk of conversion from prediabetes to new-onset diabetes (NOD) in a prospective cohort study.

Design: 205 patients with impaired fasting glucose (IFG) were followed each year during 5 years in the IT-DIAB cohort study. Twenty-one BA species and 7α-hydroxy-4-cholesten-3-one (C4), a marker of BA synthesis, were quantified by LC/MS-MS in plasma from fasted patients at baseline. Correlations between plasma BA species and metabolic parameters at baseline were assessed by Spearman's analyses and the association between BAs and NOD was determined using Cox proportional hazards models.

Results: Among the analyzed BA species, total hyocholic acid (HCA) and the total HCA/total chenodeoxycholic acid (CDCA) ratio, reflecting hepatic BA 6α-hydroxylation activity, negatively correlated with BMI and HOMA-IR. The total HCA/total CDCA ratio also correlated negatively with HbA_{1C}. Conversion from IFG to NOD occurred in 33.7% of the participants during the follow-up. Plasma BA species were not independently associated with the conversion to NOD after adjustment with classical T2D risk factors.

Conclusions: Fasting plasma BAs are not useful clinical biomarkers for predicting NOD in patients with IFG. However, an unexpected association

between 6α-hydroxylated BAs and glucose parameters was found, suggesting a role for this specific BA pathway in metabolic homeostasis.

IT-DIAB study registry number: NCT01218061.

Keywords: Bile acids, impaired fasting glucose, prediabetes, new onset diabetes, hyocholic acid, C4, HOMA-IR.

Abbreviations: 12α-hydroxylated (12α-OH); 6α-hydroxylated (6α-OH); 7α-hydroxy-4-cholesten-3-one (C4); Agence Nationale pour la Recherche (ANR); Bile acids (BAs); body mass index (BMI); chenodeoxycholic acid (CDCA); cholesterol associated with high density lipoproteins (HDL-c); cholesterol associated with low density lipoproteins (LDL-c); cholic acid (CA); CYP cholesterol 27α-hydroxylase (CYP27A1); CYP cholesterol 7α-hydroxylase (CYP7A1); cytochrome P450 (CYP); deoxycholic acid (DCA) electrochemiluminescent enzyme immunoassay (ECLIA); farnesoid X Receptor (FXR); fasting plasma glucose (FPG); glucagon-like peptide-1 (GLP-1); glycated hemoglobin (HbA_{1C}); hazard ratio (HR); high molecular weight (HMW); Homeostasis model assessment of insulin resistance (HOMA-IR); hyocholic acid (HCA); hyocholic acid (HCA); hyodeoxycholic acid (HDCA); impaired fasting glucose (IFG); insulin resistance (IR); interquartile range (IQR); lith ocholic acid (LCA); liquid chromatography tandem mass spectrometry (LC-MS/MS); methanol (MeOH); new onset diabetes (NOD); non-diabetic group (ND); Relationship between Insulin Sensitivity and Cardiovascular disease cohort (RISC); SFSTP (Société Française des Sciences et Techniques Pharmaceutiques); standard deviation (SD); Takeda G protein coupled Receptor 5 (TGR5); tandem mass spectrometry (MS/MS); total cholesterol (TC); triglycerides (TG); type 2 diabetes (T2D); ursodeoxycholic acid (UDCA).

1. INTRODUCTION

Bile acids (BAs) are steroid molecules; those synthesized in the liver are called “primary BAs”, in humans cholic acid (CA), chenodeoxycholic acid (CDCA) and hyocholic acid (HCA). BAs are conjugated either with glycine or taurine to form the glyco- or tauro-conjugated BAs, and then secreted into bile. BAs are released in the intestine, where they are deconjugated and transformed into “secondary BAs” by the gut microbiota (CA into deoxycholic acid (DCA), CDCA into ursodeoxycholic acid (UDCA) or lithocholic acid LCA (LCA), and HCA into hyodeoxycholic acid (HDCA)) **(Figure 1)**. Most BAs are recaptured in the ileum and return to the liver *via* the portal vein where they are recaptured by transporters on the sinusoidal membrane of the hepatocytes. However, a minor fraction of venous portal BAs escapes hepatic reuptake and spill over in the systemic circulation, hence reaching peripheral organs.

Besides their physicochemical role in dietary lipid solubilization and absorption, BAs signal via specific receptors, such as the Farnesoid X Receptor (FXR) and the Takeda G protein-coupled Receptor 5 (TGR5), through which they regulate not only their own hepatic synthesis, but also glucose, lipid and energy homeostasis, as well as inflammation[1,2]. Both preclinical and, mostly observational, human studies have demonstrated that BAs and their receptors are involved in the regulation of glucose metabolism by controlling, among others, insulin signaling, hepatic glucose production, glucose utilization, and the secretion of glucagon-like peptide-1 (GLP-1) (reviewed in[1,3,4]).

Quantitative and/or qualitative plasma BA alterations have been reported in obesity, insulin resistance (IR)[5], type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD)(reviewed in[6]). Whether BAs play a causal role in the

pathophysiology of the metabolic syndrome and progression to T2D remains unknown. Although BAs have been proposed as biomarkers of IR and T2D risk, their clinical utility has not been evaluated in a longitudinal study yet. To address this question, we first investigated (1) the correlations between circulating BA species and glycemic parameters in individuals with impaired fasting glucose (IFG), a prediabetic state, and (2) the predictive association of fasting plasma BA species with the conversion from IFG to new onset T2D (NOD) in a 5-year prospective cohort study (IT-DIAB study).

2. MATERIALS AND METHODS

2.1 Study population

The study population belongs to the IT-DIAB study (NCT01218061). Briefly, the IT-DIAB study is a 5-year prospective, observational study designed to identify new biomarkers of T2D risk in a population with prediabetes. The population was recruited in the occupational centers of three French cities: Nantes, Saint-Nazaire and Lille. The patient samples from Lille were not available for BA measurements and were therefore not included in the present analysis.

Written informed consent was obtained from each patient included in the study. The institutional ethics committee approved the protocol, and all the reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki (revised in 2008), as stated in a prior approval by the institution's human research committee. All patients underwent a baseline visit between June 2010 and February 2013, including a medical interview, signing of the informed consent and self-administered questionnaire of the diabetes risk score, physical examination (including body weight, height, waist and hip

circumference measurements) and blood sampling. The fatty liver index (FLI), an indirect marker of hepatic steatosis, was computed as previously described[7,8]. Patients without history of diabetes and with IFG (*i.e.* fasting plasma glucose (FPG) between 110 and 125 mg/dL [according to the WHO recommendation]) were eligible for the IT-DIAB study. The main non-inclusion criteria were: history of treatment with oral anti-diabetic agents or insulin (with the exception of gestational diabetes), severe coagulation disorder or thrombocytopenia (platelets levels <100,000/mm^3), severe renal insufficiency (defined using MDRD equation as eGFR <30mL/min.1.73m^2), severe liver impairment (prothrombin ratio <50%), severe psychiatric disorders, alcohol abuse (estimated >30 g/day), patient's opposition or inability to participate, at least, 5 years in the study.

For the present analysis, we also secondarily excluded the population for which plasma samples were not available for BA measurements (n=7) and/or without follow-up visit (n=24), with concomitant statin (n=68) and/or fibrate (n=11) therapy, and/or with baseline $HbA1_C$ ≥6.5% (n=16) or missing $HbA1_C$ (n=1). Ultimately, 205 patients with at least one follow-up visit were considered for the present analysis as shown in the flow chart (**Supplemental figure 1).**

2.2 Follow-up and conversion to new onset diabetes (NOD)

The end of the follow-up occurred at the fifth yearly visit, or prematurely if the patient met one of the following criteria: conversion to NOD, patient's withdrawal or loss to follow-up, inappropriate prescription of anti-diabetic agent (*i.e.* for prediabetes and not after the diagnosis of NOD), bariatric surgery or death.

NOD was defined by a FPG value ≥126 mg/dL or a plasma glucose ≥200 mg/dL after 2-hour oral glucose tolerance test.

2.3 Biochemical analyses

During the baseline visit, peripheral venous blood samples for biological analysis were obtained in the morning after overnight fasting. Standard biological analyses included FPG and HbA_{1C}. Frozen heparinized plasma was used for insulin measurement by electrochemiluminescent enzyme immunoassay (ECLIA), using the Cobas e automated clinical analyzer system (Roche Diagnostics, Meylan, France). Plasma high molecular weight (HMW) adiponectin levels were measured by ECLIA on an automated clinical analyzer system Lumipulse G600 (Fujirebio, Les Ulis, France). Homeostasis model assessment of insulin resistance (HOMA-IR) was defined according to the equation proposed by Matthews *et al.*[9].

2.4 Plasma bile acids and C4 quantification

21 BA species concentrations (**Supplemental table 1**) and 7alpha-hydroxy-4-cholesten-3-one (C4) concentration were quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after extraction from plasma by protein precipitation as previously described [10].

2.5 Statistical analyses

Categorical variables are presented using population size (%) and related between-group comparisons were tested using Fisher's exact test. Quantitative variables are presented using mean ± standard deviation (SD) or median

(interquartile range, IQR) in case of skewed distribution, with appropriate comparison tests for non-paired series (respectively, Student's T-test or Wilcoxon rank-sum test). The association between two quantitative variables is presented using the Spearman's R correlation coefficient.

For the longitudinal analysis, Kaplan-Meier survival curves were drawn using conversion to NOD as the event of interest, and the terciles of the distribution of different explanatory variables to define the studied groups. The association between the different BA species and the conversion to NOD was also studied using a Cox model based on the proportional hazard hypothesis, before and after adjustment on the classical risk factors for T2D: age, BMI, FPG and HbA_{1C}.

A p value <0.05 was deemed statistically significant. All analyses were performed using R software version 3.5.1 (The R Foundation for Statistical Computing, R Core Team, Vienna, 2018), with the RStudio interface.

3. RESULTS

3.1 Study participants

The baseline clinical-biological parameters of the studied population are shown in **Table 1**. In accordance with the inclusion criteria, all participants had prediabetes at baseline with IFG (mean FPG ± SD: 116 ± 4 mg/dL) and a median $HbA1_C$ at 5.8% (39.9 mmol/mol). The participants were middle aged (mean age ± SD: 56.0 ± 9.9 years), overweight (mean BMI ± SD: 29.4 ± 6.3 kg/m^2), with 68.8% males. Participants had insulin resistance with a mean ± SD HOMA-IR at 3.82 ± 2.74 and NAFLD with median FLI >60% (median, [IQR]: 62.4 [36.0-83.4]).

3.2 Cross-sectional associations between plasma bile acid species and metabolic parameters at baseline

As a first approach to investigate the link between BA metabolism and glucose homeostasis, we assessed the correlations between plasma BA species and the metabolic parameters in the IT-DIAB participants at baseline (**Table 2**; significant correlations are plotted in **Supplemental Figure 2**). The absolute concentrations (nM) of the BAs are presented in the **Supplemental Table 2**.

Among the different BA species and ratios, total concentrations of 6α-hydroxylated HCA negatively correlated with BMI, HOMA-IR and FLI, suggesting a role for 6α-hydroxylation in the pathophysiology of obesity, insulin resistance and NAFLD. These correlations were even more significant for the total HCA/total CDCA ratio, which likely reflects the rate of hepatic 6α-hydroxylation of CDCA to HCA (see **Figure 1**), which also negatively correlated with HbA_{1C} (**Table 2**). Inversely, plasma C4 levels were significantly and positively associated with BMI, HOMA-IR and FLI. There was also a significant inverse correlation between C4 and total HCA ($R=-0.33$, $p<0.0001$) (**Supplemental Figure 3**), indicating that alterations in 6α-hydroxylation may possibly mechanistically interact with the classical synthesis pathway (initiated by CYP7A1; **Figure 1**). In contrast, plasma BAs, total CA, total CDCA and the 12α-OH/non-12α-OH BA ratio were not significantly correlated with markers of glucose homeostasis. The correlations between the metabolic parameters and the individual BA species are listed in **Supplemental Table 3**.

3.3 Plasma bile acid species and conversion to type 2 diabetes

Among the 205 patients with prediabetes enrolled in this study, 69 (33.7%)

converted to NOD after a median follow-up of 59.6 months (min: 0.7 - max: 73.1). To further determine the potential clinical relevance of plasma BAs as biomarkers of T2D risk, we analyzed whether baseline BAs predict the risk of conversion to NOD in this population.

Interestingly, Kaplan-Meier survival analysis shows that patients in the upper third tertile of the ratio of total HCA/total CDCA displayed a significantly lower risk of conversion to NOD (p=0.0039) (**Figure 2).** As expected, classical risk factors for T2D (BMI, FPG, HbA_{1C} and HOMA-IR) **(Table 3)** significantly associated with NOD conversion in both univariate and multivariate Cox models. Although the total HCA/total CDCA ratio was significantly associated with NOD conversion in the univariate Cox model (HR: 0.78 [0.61; 1.00]; p=0.048), this association was no longer significant after adjustment to classical risk factors for T2D (**Table 3**). Plasma total HCA and C4 concentrations also failed to be significantly associated with the conversion to NOD (HR: 0.84 [0.65; 1.08] and 1.17 [0.93; 1.48], respectively). None of the other plasma BA parameters (total plasma BAs (including total free, total conjugated, total primary and total secondary BAs), total CA, total CDCA, total DCA concentrations) nor the 12α-OH/non-12α-OH BA ratio were associated with the risk of NOD, both in univariate and multivariate Cox models.

4. DISCUSSION

This is, to the best of our knowledge, the first longitudinal study assessing the clinical relevance of plasma BAs as biomarkers of T2D risk in a population with prediabetes. Our results indicate that peripheral BAs do not predict the transition from IFG to NOD. Indeed, in our cohort, baseline plasma

concentrations of the different BA species were not independently associated with the risk of NOD after a 5-year longitudinal follow-up. In addition, qualitative plasma BA parameters, such as the 12α-OH/non-12α-OH (previously reported to reflect hepatic insulin resistance[11]) or conjugated/free BA ratios, did not predict NOD conversion.

It is noteworthy that the total HCA/total CDCA ratio, which likely reflects the rate of hepatic 6α-hydroxylation of CDCA to form HCA, predicted the risk of NOD in univariate survival analyses. However, the predicting value of the total HCA/total CDCA ratio did not persist after adjustment for classical T2D risk factors (age, BMI, FPG and HbA_{1C}). Nevertheless, BAs co-segregate with metabolic alterations in prediabetes suggesting an upstream role of BAs in the control of glucose metabolism. This is supported by a wealth of preclinical data showing that BA modulate glucose metabolism by signaling through their receptors FXR and TGR5 and clinically since BA sequestrants are efficacious agents in the treatment of diabetes[12]. Accordingly, a negative correlation of baseline total HCA concentrations was observed with BMI, insulin resistance (*i.e.* HOMA-IR), and FLI. HOMA-IR and BMI associate with FLI, as expected, since FLI is a good predictor of NAFLD, and since NAFLD is a common co-morbidity of the metabolic syndrome. Therefore, our results show that although BAs do not independently predict NOD, 6α-hydroxylated (6α-OH) BAs negatively correlate with metabolic alterations predisposing to T2D, suggesting a potential pathophysiological link between glucose metabolism and hepatic 6α-hydroxylation.

The synthesis routes of 6α-OH HCA (also known as γ-muricholic acid) differ between mammal species. Although 6α-hydroxylation of BAs in humans has not

been extensively studied, it is known that HCA is produced in hepatocytes from CDCA *via* human hepatic CYP3A4[13]. The proportion of HCA in the human BA pool is minor in comparison with the major BA species, and for this reason HCA is often neglected in human plasma BA analyses notwithstanding its high concentrations in human fetal bile[14]. Our data revealed a negative correlation of BMI and HOMA-IR with total HCA due to conjugated HCA species, in line with previous reports[15,16]. Of note, CYP3A4 is one of the most prominent human CYP enzymes and its activity can be modulated by several endogenous and exogenous factors[17]. Since a signaling role of 6α-OH BAs has not yet been identified, it is conceivable that these BA species are rather reflecting a metabolic state of altered CYP3A4 activity affecting glucose metabolism. In accordance with this hypothesis, previous studies reported an association between a polymorphism in *CYP3A4* and the risk of T2D in a Japanese population[18] and reduced expression and activity of CYP3A4 in livers from diabetic donors[19,20]. Interestingly, HCA and its secondary intestinal microbiota-derived HDCA species are the major BA species in pigs[21], an animal model highly resistant to the induction of insulin resistance and diabetes. Whether 6α-OH BAs act as markers or actors in the pathophysiology of glucose homeostasis disorders requires further study.

While concentrations of plasma C4, a marker of the rate of the classical BA synthesis pathway, failed to be significantly associated with the risk of NOD, it was positively correlated with both BMI and HOMA-IR, as previously described[22,23]. Conversely, plasma C4 levels negatively correlated with adiponectin, suggesting a possible link between the classical BA synthesis pathway and insulin resistance. Interestingly, C4 positively associated with FLI,

in line with the suggested role of BAs in hepatic fat metabolism through the activation of FXR (ref1)[24].

In the RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) cohort, the 12α-OH/non-12α-OH BA ratio was associated with insulin sensitivity[25]. Surprisingly, the 12α-OH/non-12α-OH BA ratio was neither associated with HOMA-IR nor with the risk of NOD in the IT-DIAB study. This divergence could be due to the differences in the range of insulin resistance between the patients from IT-DIAB and those in the previously published studies, since our study included only individuals with prediabetes (*i.e.* impaired fasting glycaemia), whereas the RISC cohort included a broader range of patients. Furthermore, we assessed insulin resistance by calculating HOMA-IR, whereas it was assessed during hyperinsulinemic euglycemic clamps in the RISC cohort.

The main strengths of our study are the prospective design and the duration of the follow-up, with substantial numbers of patients converting to NOD, and the quality and the frequency of the data collection, including a consultation with a physician and biological follow-up at least on a yearly basis, allowing timely detection of the primary outcome. Also, complete and precise assessment of the BA profiles with 21 species, as well as C4, was obtained. Our study presents some limitations however. Only fasting plasma BA samples were measured and post-prandial data are lacking. The observational design of the study allows us to conclude on associations, but not on causality. A the time of the study design, we selected WHO diagnostic criteria for prediabetes and IFG rather than the most recent *American Diabetes Association* (ADA)

guidelines[26]. Finally, the exploratory correlation analysis and the multiplicity of testing did not take into account the α-risk inflation.

5. CONCLUSIONS

In conclusion, while quantitative and qualitative alterations in BA metabolism are associated with metabolic parameters, most plasma BA species are not useful biomarkers to predict NOD in patients with IFG. The role of the 6α-hydroxylation pathway (*via* CYP3A4, leading to HCA synthesis) in the pathophysiology of T2D is intriguing and requires further study.

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Duality of interest

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CONTRIBUTIONS

BS and BC designed the study. MW performed the statistical analysis. MW, MP and MJ collected the data. OCT, AD, EV, MK, EBC and JFG performed the LC-MS and biochemical analyses. MW, MP, CLM, RH and SH contributed to the discussion and reviewed the manuscript. OCT, MW, AT, BS and BC wrote the first draft, edited and reviewed the manuscript. MW and BC are investigators of the IT-DIAB study. All the authors have read and approved the final version of the manuscript. BC is the guarantor of this study.

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Table 1.

	N=205	Available data
Clinical characteristics		
Age (y)	56.0 ± 9.9	205/205
Sex (female)	64 (31.2 %)	205/205
BMI (kg/m²)	29.4 ± 6.3	205/205
Diabetes risk score	13.9 ± 4.7	205/205
Waist circumference (cm)	97.3 ± 14.2	203/205
Hip circumference (cm)	104.8 ± 13.3	201/205
Waist:hip ratio	0.93 ± 0.08	201/205
Lipid profile		
Total cholesterol (mg/dL)	225.3 ± 38.5	205/205
Triglycerides (mg/dL)	133.9 ± 81.7	204/205
LDL-c (mg/dL)	144.6 ± 35.1	201/205
HDL-c (mg/dL)	53.7 ± 15.7	204/205
Non-HDL-c (mg/dL)	171.2 ± 41.0	204/205
Glucose homeostasis		
FPG (mg/dL)	116 ± 4	205/205
HbA_{1c} (%)	5.8 (5.5-6.0)	205/205
HbA_{1c} (mmol/mol)	39.9 (36.6-42.1)	205/205
Insulin (mUI/L)	10.6 (7.2-16.4)	189/205
HOMA-IR	3.82 ± 2.74	189/205
Adiponectin (µg/L)	3.94 ± 2.27	190/205
Fatty liver index	62.4 (36.0-83.4)	199/205

Table 1. Baseline characteristics of the studied population.

Legend: Data are presented as population size (%), mean ± SD or median (25^{th} – 75^{th} percentile), according to their distribution.

Body mass index (BMI); FPG: fasting plasma glucose; Homeostasis model assessment of insulin resistance (HOMA-IR).

Table 2

	Spearman's correlation coefficient					
	BMI	**WHR**	**FPG**	**HbA_{1c}**	**HOMA-IR**	**FLI**
Total BAs	0.01	0.07	0.05	0.04	0.09	0.08
Total free BAs	0.04	**0.15***	0.07	0.02	0.09	0.13
Total glyco-conjugated BAs	-0.02	0.03	0.07	0.01	0.07	0.07
Total tauro-conjugated BAs	0.04	-0.12	0.09	0.02	**0.18***	0.07
Total CA	0.05	0.04	0.07	0.05	0.12	0.05
Total CDCA	0.03	0.06	0.03	0.09	0.11	0.07
Total DCA	0.0	0.06	0.12	-0.03	0.05	0.05
Total LCA	-0.10	-0.01	0.08	-0.10	0.03	-0.09
Total UDCA	0.01	0.12	0.01	-0.02	0.04	0.09
Total HCA	**-0.20****	-0.01	0	-0.11	**-0.16***	**-0.15***
Total HDCA	-0.06	0.05	0.06	-0.06	-0.04	0.01
Primary/Secondary BA ratio	0.01	-0.06	-0.04	0.10	0.07	-0.07
Conjugated/Free BA ratio	-0.07	**-0.15***	0.01	-0.02	-0.03	-0.09
12αOH/non-12αOH BA ratio	0.03	-0.01	0.07	-0.06	-0.06	0.03
Total HCA/Total CDCA ratio	**-0.27*****	-0.06	-0.05	**-0.20****	**-0.30*****	**-0.25*****
C4	**0.32*****	0.14	-0.05	0.11	**0.34*****	**0.35*****

Table 2. Correlation between bile acids and the clinical-biological parameters of the studied population at baseline.

Legend: Spearman's correlation coefficient (R) is presented for each pair of parameters. Significant values are presented in bold: *p<0.05, **p<0.01 or ***p<0.001. BA clusters are defined in the **Supplemental Table 1**. BAs: bile acids; BMI: body mass index; C4: 7α-hydroxy-4-cholesten-3-one; FLI: Fatty Liver Index; FPG: fasting plasma glucose; HOMA-IR: homeostasis model assessment for insulin resistance; WHR: waist-hip ratio.

Table 3

	HR (95%CI)	
	Not adjusted	**Adjusted**
Adjustment factors		
Age (+1 SD)	1.14 (0.88; 1.48)	
BMI (+1 SD)	**1.32 (1.08; 1.61)****	
FPG (+1 SD)	**1.62 (1.28; 2.06)*****	
HbA_{1c} (+1 SD)	**1.66 (1.26; 2.19)*****	
Bile acid clusters tested‡		
Total BAs (+1 SD)	0.99 (0.78; 1.26)	0.93 (0.71; 1.22)
Total free BAs (+1 SD)	0.99 (0.78; 1.26)	0.96 (0.74; 1.24)
Total glyco-conjugated BAs (+1 SD)	1.05 (0.84; 1.33)	1.00 (0.78; 1.27)
Total tauro-conjugated BAs (+1 SD)	0.94 (0.73; 1.22)	0.89 (0.67; 1.18)
Total CA (+1 SD)	0.97 (0.76; 1.23)	0.93 (0.70; 1.23)
Total CDCA (+1 SD)	0.98 (0.77; 1.25)	0.90 (0.70; 1.17)
Total DCA (+1 SD)	1.07 (0.84; 1.36)	1.00 (0.79; 1.26)
Total LCA (+1 SD)	0.96 (0.75; 1.22)	0.98 (0.75; 1.28)
Total UDCA (+1 SD)	1.05 (0.85; 1.30)	1.09 (0.88; 1.36)
Total HCA (+1 SD)	0.84 (0.65; 1.08)	0.89 (0.68; 1.16)
Total HDCA (+1 SD)	0.72 (0.40; 1.31)	0.73 (0.36; 1.49)
Primary/Secondary BA ratio (+1 SD)	0.86 (0.66; 1.11)	0.81 (0.62; 1.07)
Conjugated/Free BA ratio (+1 SD)	0.92 (0.71; 1.19)	0.91 (0.70; 1.18)
12αOH/non-12αOH BA ratio (+1 SD)	1.01 (0.80; 1.27)	1.01 (0.80; 1.27)
Total HCA/Total CDCA ratio (+1 SD)	**0.78 (0.61; 1.00)***	0.88 (0.67; 1.16)
C4 (+1 SD)	1.17 (0.93; 1.48)	1.08 (0.84; 1.37)
HOMA-IR (+1 SD)	**1.41 (1.17; 1.69)*****	**1.33 (1.03; 1.72)****

Table 3. Association between bile acid clusters at baseline and the conversion to new onset diabetes during the 5-year follow-up.

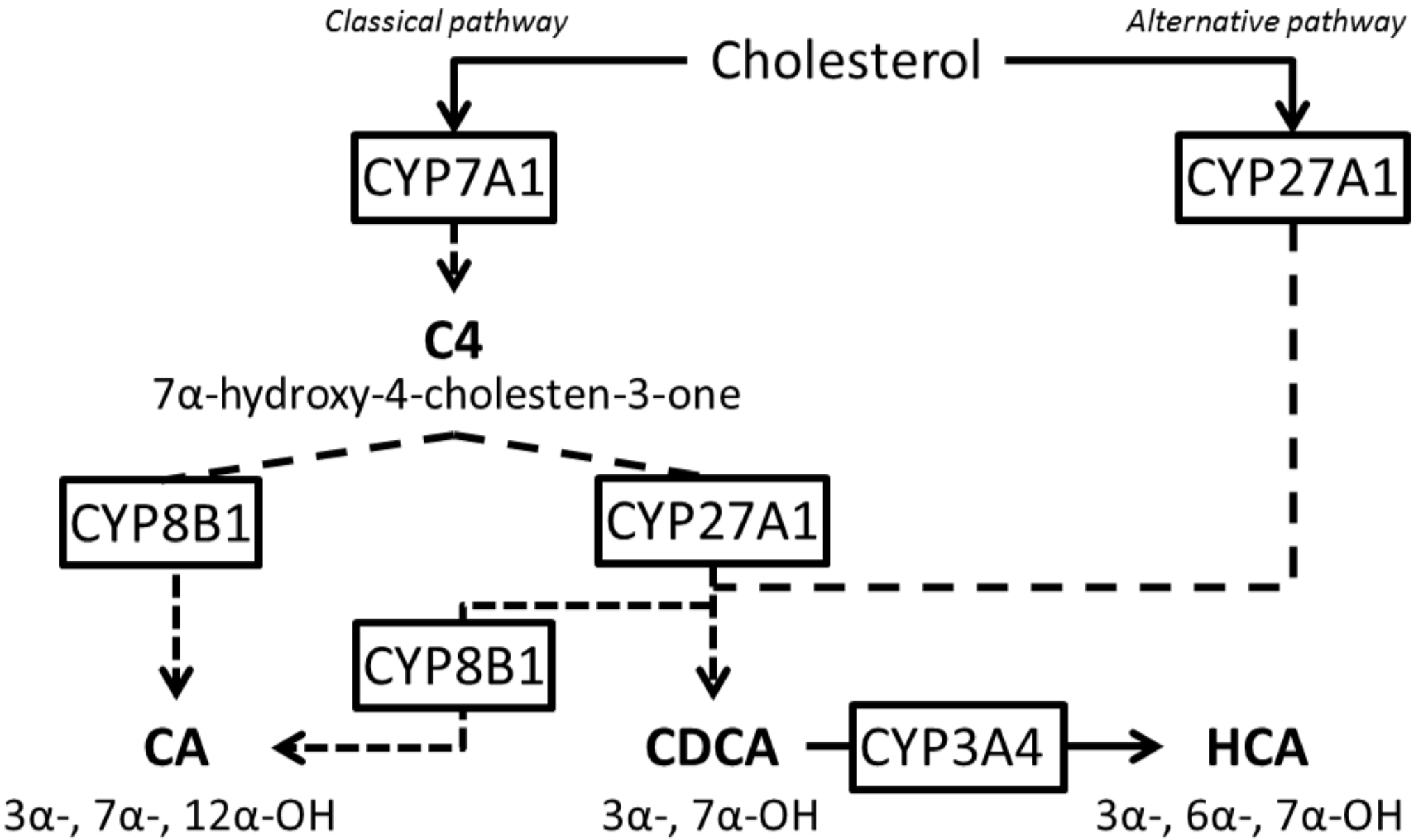
Legend: Cox proportional hazards models before and after adjustment on baseline values for age, BMI, fasting plasma glucose and HbA_{1C}. No significant interaction was found between sex and the other parameters of interest. The multivariate model with HOMA-IR was not adjusted on baseline FPG because of obvious co-linearity. ‡ To better fit with the model, all BA values were transformed using square root function except for total HDCA. Significant values displayed as: *p<0.05, **p<0.01 or ***p<0.001. BA clusters are defined in the **Supplemental Table 1**. BAs: bile acids; C4: 7α-hydroxy-4-cholesten-3-one; FPG: fasting plasma glucose; HOMA-IR: Homeostasis model assessment for insulin resistance; Data shown as HR (95% CI): Hazard-Ratio with 95% confidence interval.

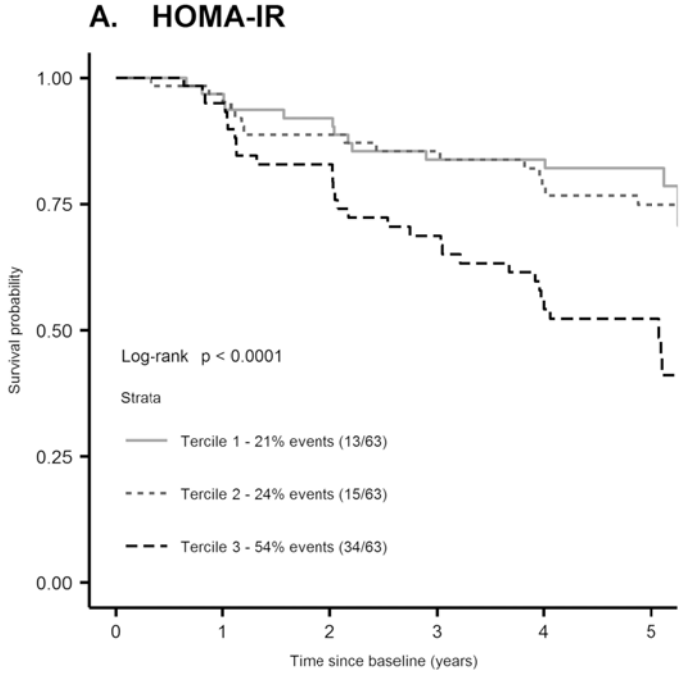
Figure 1. Primary bile acid synthesis pathways.

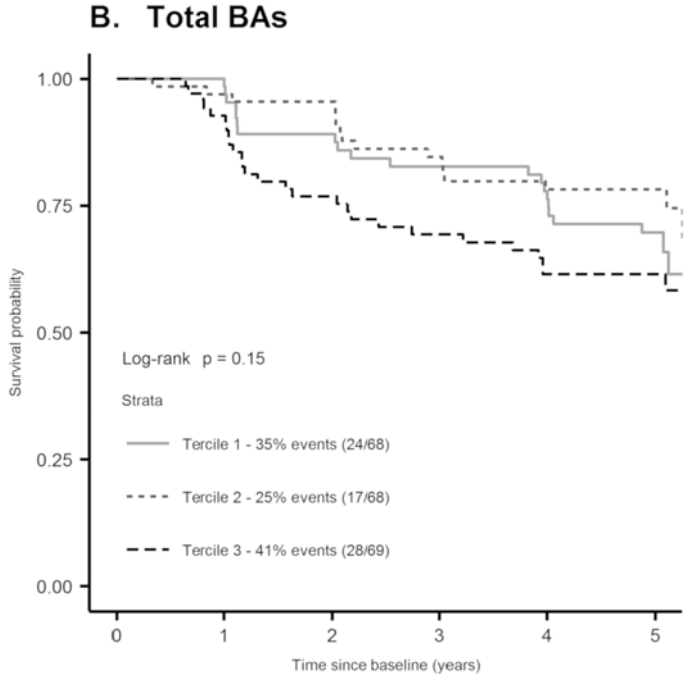
Legend: Primary BAs are synthesized from cholesterol within hepatocytes by two pathways. The classical pathway synthesizes most of BAs and its rate-limiting enzyme is the CYP7A1, whose synthesis rate biomarker is the metabolite C4. The alternative pathway's rate-limiting enzyme is the CYP27A1. The end products of these pathways are the primary BAs: CA is a 3α-, 7α-, 12α-OH BA whose synthesis is mediated by the 12α-hydroxylase CYP8B1. CDCA is 3α-, 7α-OH and is produced via both the classical and the alternative BA synthesis pathways via the enzyme CYP27A1. HCA is 3α-, 6α-, 7α-OH and is synthesized upon 6α-hydroxylation of CDCA via the CYP3A4.

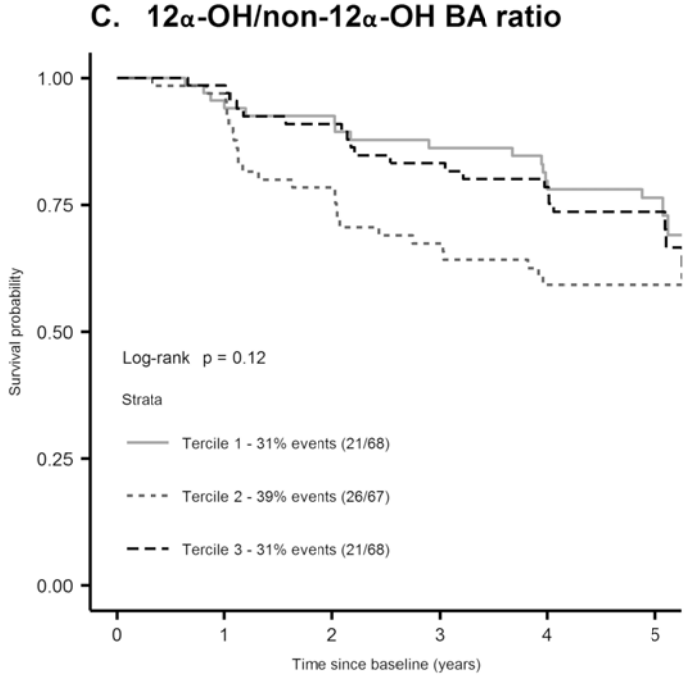
Figure 2. Survival curves for conversion from prediabetes to type 2 diabetes in the IT-DIAB study.

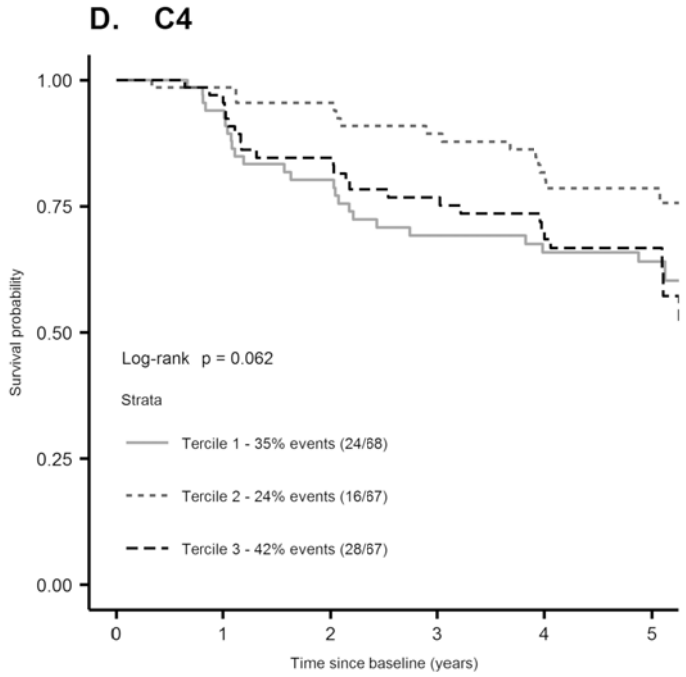
Legend: Survival curves for conversion to new onset diabetes according to HOMA-IR (A), total bile acids (B), 12αOH/non12αOH BA ratio (C), C4 (D), total HCA (E) and total HCA/total CDCA ratio (F)**.** Kaplan-Meier's survival curves, each parameter being presented by groups created using tertiles as thresholds (T1 < first tertile < T2 < second tertile < T3). BA clusters are defined in the **Supplemental table 1**.

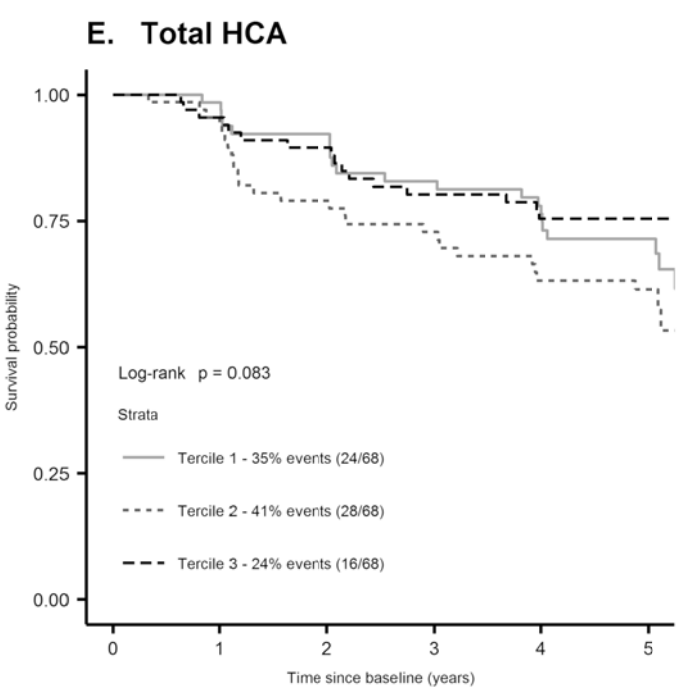

Classical pathway
Alternative pathway
Cholesterol
CYP7A1
CYP27A1
C4
7α-hydroxy-4-cholesten-3-one
CYP8B1
CYP27A1
CYP8B1
CA
CDCA
CYP3A4
HCA
3α-, 7α-, 12α-OH
3α-, 7α-OH
3α-, 6α-, 7α-OH

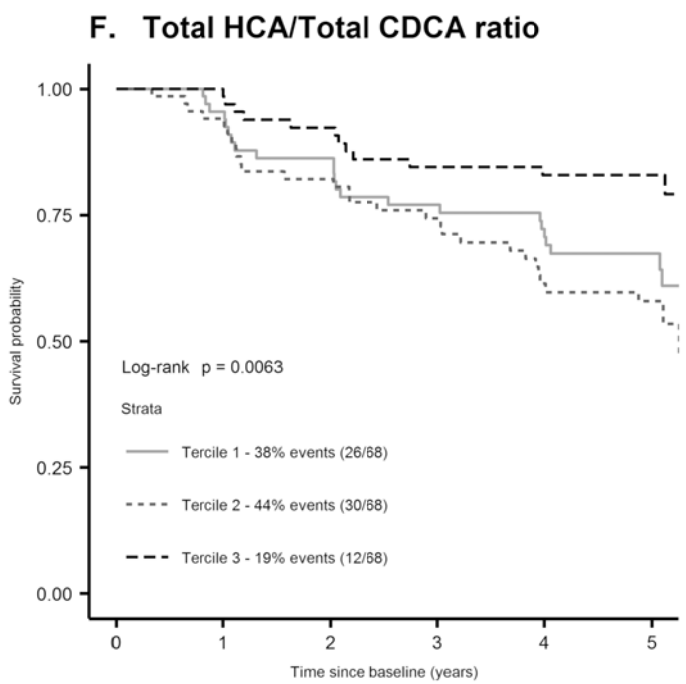

A. HOMA-IR
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank p < 0.0001
Strata
Tercile 1 - 21% events (13/63)
Tercile 2 - 24% events (15/63)
Tercile 3 - 54% events (34/63)
0
1
2
3
4
5
Time since baseline (years)


B. Total BAs
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank p = 0.15
Strata
Tercile 1 - 35% events (24/68)
Tercile 2 - 25% events (17/68)
Tercile 3 - 41% events (28/69)
0
1
2
3
4
5
Time since baseline (years)


C. 12α-OH/non-12α-OH BA ratio
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank p = 0.12
Strata
Tercile 1 - 31% events (21/68)
Tercile 2 - 39% events (26/67)
Tercile 3 - 31% events (21/68)
0
1
2
3
4
5
Time since baseline (years)


D. C4
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank p = 0.062
Strata
Tercile 1 - 35% events (24/68)
Tercile 2 - 24% events (16/67)
Tercile 3 - 42% events (28/67)
0
1
2
3
4
5
Time since baseline (years)


E. Total HCA
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank p = 0.083
Strata
Tercile 1 - 35% events (24/68)
Tercile 2 - 41% events (28/68)
Tercile 3 - 24% events (16/68)
0
1
2
3
4
5
Time since baseline (years)


F. Total HCA/Total CDCA ratio
Survival probability
1.00
0.75
0.50
0.25
0.00
Log-rank p = 0.0063
Strata
Tercile 1 - 38% events (26/68)
Tercile 2 - 44% events (30/68)
Tercile 3 - 19% events (12/68)
0
1
2
3
4
5
Time since baseline (years)