UiT The Arctic University of Norway

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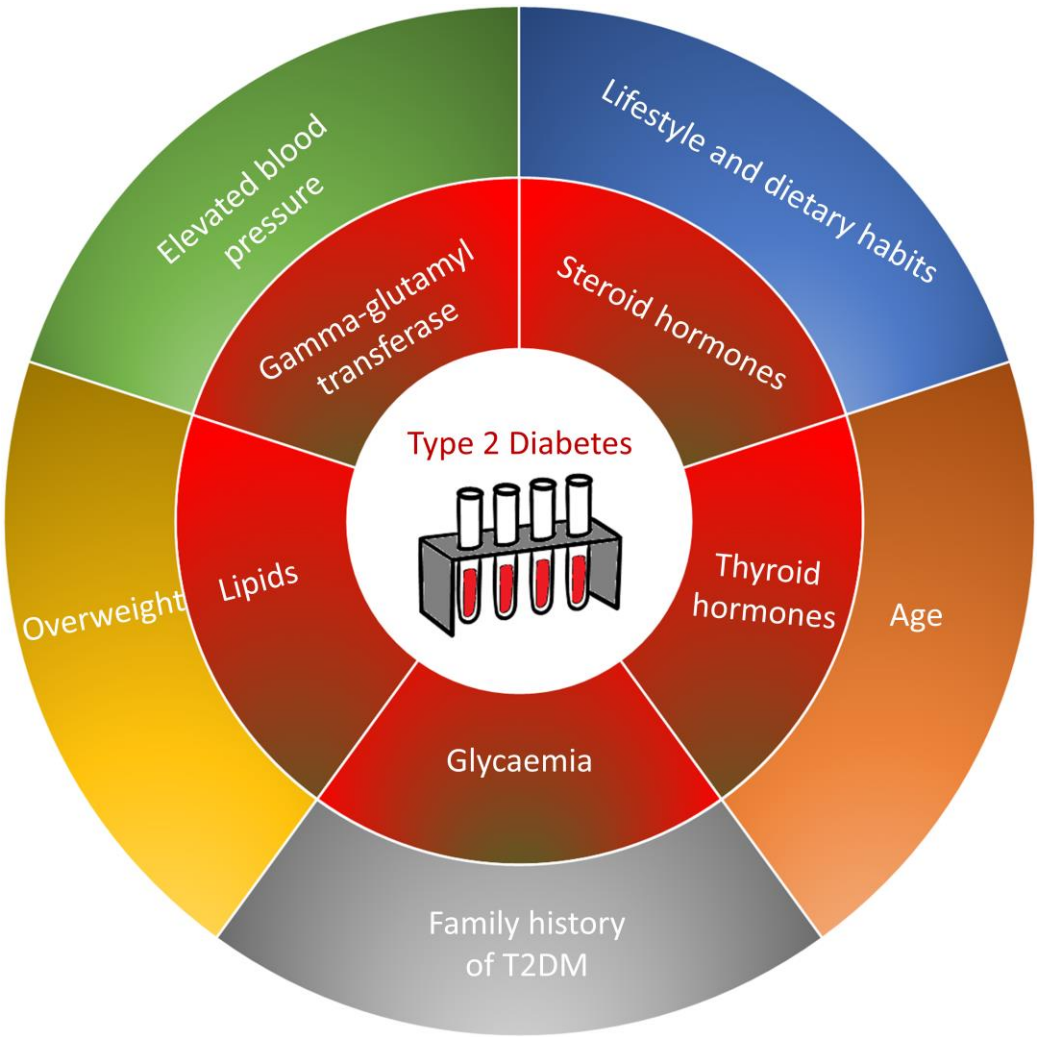
Blood biomarkers and Type 2 Diabetes Mellitus

Repeated measurements of blood biomarkers in Type 2 Diabetes Mellitus cases and controls; longitudinal assessments and associations

Giovanni Allaoui

A dissertation for the degree of Philosophiae Doctor – July 2023



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Abbreviations

11KA4, 11-ketoandrostenedione

11KT, 11-ketotestosterone

11OHT, 11-hydroxytestosterone

11OHA4, 11-hydroxyandrostenedione

25(OH)D, 25-hydroxyvitamin D

A4, Androstenedione

AIC, Akaike's information criterion

AKR1C3, Enzyme aldo-ketoreductase type 1 C3

AROC, Area under the receiver operating characteristic curve

AUC, Area under the curve

BMI, Body mass index

CI, Confidence interval

CV, Coefficient of variation

CYP11B1, Cytochrome P450 family 11 subfamily B member 1

DAG, Directed acyclic graph

DHEAS, Dehydroepiandrosterone sulphate

HbA_{1c}, Glycated haemoglobin

HDL-C, High-density lipoprotein cholesterol

GGT, Gamma-glutamyl transferase

IQR, Inter quartile range

LC-MS/MS, Liquid chromatography tandem mass spectrometry

LDL-C, Low-density lipoprotein cholesterol

LLE, Liquid-liquid extraction

LR-test, Likelihood-ratio test

OR, Odds ratio

ROC, Receiver operating characteristic

SD, Standard deviation

T1-T3, Time-point 1 – Time-point 3

T_3, Triiodothyronine

T_4, Thyroxine

T2DM, Type 2 diabetes mellitus

TSH, Thyroid stimulating hormone

List of papers

Paper I

Allaoui G, Rylander C, Averina M, Wilsgaard T, Fuskevåg OM, Berg V (2022) Longitudinal changes in blood biomarkers and their ability to predict type 2 diabetes mellitus-The Tromsø study. Endocrinol Diabetes Metab 5(2): e00325. DOI: 10.1002/edm2.325

Paper II

Allaoui G, Rylander C, Fuskevåg OM, Averina M, Wilsgaard T, Brustad M, Jorde R, Berg V (2023) Longitudinal changes in vitamin D concentrations and the association with type 2 diabetes mellitus: the Tromsø Study. Acta Diabetol 60, 293–304. DOI: 10.1007/s00592-022-02001-y

Paper III

Allaoui G, Rylander C, Fuskevåg OM, Grimnes G, Averina M, Wilsgaard T, Berg V (2023) Longitudinal assessment of classic and 11-oxygenated androgen concentrations and their association with the development of type 2 diabetes – the Tromsø Study. Manuscript (submitted).

Abstract

Background

The prevalence of diabetes is increasing worldwide and is estimated to be 10.5% in 2021, with over 90% of the patients diagnosed with type 2 diabetes mellitus (T2DM). T2DM is a chronic metabolic disease characterised by insulin resistance and insufficient insulin secretion leading to dysglycaemia. As such, the disease progresses slowly over time, but once T2DM is developed it is difficult to treat and is associated with cardiovascular complications, degradation of nerve fibres and eyesight, and impaired kidney function. However, not all individuals in the early stages of dysglycaemia progress to T2DM, and the onset of the disease may be prevented or delayed by understanding the underlying susceptibility to T2DM. Therefore, identifying early predictors of T2DM is particularly interesting.

Aims

The aims of this thesis were to i) investigate blood biomarkers related to metabolic processes (lipid, glucose, and hormone homeostasis) and their association with T2DM; ii) compare pre- and post-diagnostic concentrations of blood biomarkers between T2DM cases and healthy controls; and iii) assess the efficacy of the biomarkers as potential early predictors of T2DM beyond established risk factors for the disease.

Materials and methods

This longitudinal nested case-control study, based on the Tromsø Study health surveys, included participants who attended three to five surveys (time-point 1–5; T1–T5) in the period 1986–2016. Blood biomarkers related to lipid, glucose, and hormone homeostasis were analysed in thawed serum samples. Linear mixed models and generalised estimating equations were used to compare changes over time between cases and controls. Logistic regression models were used to estimate odds ratios (ORs) for T2DM and the area under the receiver operating characteristics curve (AROC) was used to assess discrimination between cases and controls.

Results

Differences in the concentrations were observed between cases and controls for androgens, thyroid hormones, glycaemic biomarkers, gamma-glutamyl transferase (GGT), lipids, and 25-hydroxyvitamin D (25(OH)D) in both men and women during the study period. Cases

experienced a deterioration in concentrations of 25(OH)D, GGT, and glycaemic biomarkers before diagnosis, followed by an improvement in concentrations of 25(OH)D and lipids, but a deterioration in GGT and glycaemic biomarkers after diagnosis, compared to the control groups. In women, free triiodothyronine (T_3), glycaemic biomarkers, GGT, total lipids, and triglycerides were significantly and positively associated with T2DM before diagnosis. Additionally, the 25(OH)D, high-density lipoprotein cholesterol (HDL-C), and free thyroxine (T_4) were significantly and inversely associated with T2DM before diagnosis. Similarly, in men, 11-oxygenated androgens, 25(OH)D, free T_3, GGT, glycated haemoglobin (HbA_{1c}), and lipids were significantly and positively associated with T2DM whereas testosterone was significantly and inversely associated with T2DM before diagnosis. A prediction model integrating GGT, HbA_{1c}, and HDL-C (in the case of women) in conjunction with established non-invasive risk factors, demonstrated the highest discriminatory ability seven years before the diagnosis (AROC: 0.95 for women, 0.85 for men).

Conclusion

Our study findings highlighted notable associations between several blood biomarkers implicated in lipid, glucose, and hormone homeostasis and the risk of developing T2DM long before the diagnosis, among both men and women. By utilising a combination of lipids and glycaemic biomarkers in addition to established non-invasive risk factors, it was possible to successfully identify individuals with an increased risk of developing T2DM as early as 15 years before the diagnosis. We observed notable differences in the concentrations of 11-oxygenated androgens between the cases and controls, which, to the best of our knowledge, has not been previously reported. Given the limited understanding of the association between androgens and T2DM, these findings highlight the need for further investigation. Furthermore, although concentrations of thyroid hormones and vitamin D were associated with disease progression, they were found to be inadequate predictors of T2DM.

1 Introduction

1.1 Type 2 Diabetes Mellitus

Diabetes mellitus encompasses a group of diseases related to perturbations in glucose metabolism that lead to chronic hyperglycaemia owing to dysfunctional insulin homeostasis [1]. Type 2 diabetes mellitus (T2DM) is the most common type of diabetes mellitus, accounting for more than 90% of all diabetes cases [2]. It is a slow-progressing disease, which is preceded by prediabetes, and is characterised by beta cell dysfunction, impaired insulin secretion, and insulin resistance [2, 3]. Once T2DM is established, it is irreversible and difficult to manage because of the extensive deterioration of beta cells [4-7]. Thus, identifying individuals at a high risk of developing T2DM or prediabetes will enable the initiation of early interventions to prevent or delay the disease and reduce the burden on healthcare systems [1, 5, 8]. Age, body mass index (BMI), physical activity, elevated blood pressure, and family history of T2DM are well-established non-invasive risk factors commonly utilised for risk assessment and subsequent prevention of the disease [9, 10]. However, there is a rising interest in exploring the potential of blood biomarkers to identify individuals at a high-risk in the early stages. Additionally, gaining a comprehensive, understanding of disease progression is an area of significant interest [10]. Repeated measurements of blood biomarkers taken before the diagnosis in individuals who later developed T2DM as well as in healthy controls who remained disease-free, it becomes possible to identify potential early disturbances in metabolic processes.

1.1.1 Epidemiology

Diabetes ranks among the top ten leading causes of death, worldwide. The global prevalence of diabetes in the general population was estimated to be 10.5% in 2021 and is projected to increase to 11.3% by 2030 and 12.2% by 2045 [2, 11]. Its prevalence has steadily increased since an estimated prevalence of 4.6% in the year 2000 [12]. Currently, it is estimated that 45% of individuals living with diabetes worldwide remain undiagnosed, with a specific rate of 33.5% reported in Norway [2]. The global economic burden of diabetes as of 2021 and its consequences (leading to, for instance, loss of work/wages and increased cost of interventions) amounts to 966 billion USD for patients, health systems, and the nation as a

whole [2]. Specifically in Norway, the prevalence of diabetes in 2015 was estimated to be 3.7% of the population (294,000 individuals). The disease was identified as the cause of death for 862 individuals [13]. In 2021, its prevalence in Norway increased to 4.8%, with an estimated health expenditure of 11,166 USD per individual [2]. Although the prevalence of diabetes is on the rise, the incidence of the disease has witnessed a decline or stabilisation over the past decade. This encouraging trend can be attributed to multi-stage prevention efforts aimed at high-risk individuals, the general population, and the implementation of nation-wide policies [2, 14, 15]. The increased prevalence of the disease may be attributed to an aging population, a more sedentary lifestyle, increased dietary intake of unhealthy foods, and an overall increase in body weight [2, 16]. Unhealthy lifestyle habits have also been observed to increase the prevalence of T2DM in children [2]. Another reason for the increased prevalence of T2DM could potentially be the decline in the percentage of undiagnosed individuals as a consequence of increased efforts in risk assessment and frequency of HbA_{1c} measurements, which may have also increased awareness of T2DM since it was recommended as a diagnostic biomarker in 2011 [17, 18]. A longitudinal study with repeated measurements conducted by Langholz et al. [19] on the general population from the Tromsø municipality indicated that the number of undiagnosed individuals declined from 32 to 17% for women and from 37 to 24% for men over a 22 year time-period, from 1994 to 2016.

1.1.2 Pathogenesis

Blood glucose is primarily regulated by insulin and glucagon, which are hormones secreted by the pancreatic beta and alpha cells, respectively [20, 21]. These hormones help in regulating the glucose levels in the body, whereby an increase in glucose levels induces the production of insulin which stimulates glucose uptake in target tissues, whereas a decrease in glucose levels induces the production of glucagon which stimulates hepatic glucose production [22]. In addition, insulin and glucagon production is regulated by the insulin-to-glucagon ratio [23, 24]. The development of T2DM is characterised by the progressive deterioration of beta cell function, which commences with prediabetes that includes impaired fasting glucose levels and glucose tolerance, which eventually leads to the deterioration of beta cells and the manifestation of insulin resistance resulting in hyperglycaemia [22]. Beta-

cell mass is believed to be reduced by 20–40% in individuals with prediabetes and reduced by 30–65% in individuals diagnosed with T2DM [25]. A progressive decline in insulin regulation and resistance has been observed for up to six years before individuals eventually developed T2DM, with evident differences between cases and controls for up to 13 years before the diagnosis of the disease [26, 27]. Obesity is a major contributor to the development of T2DM, which induces low-grade inflammation and can lead to beta-cell dysfunction and insulin resistance [1, 28, 29]. Obesity also increases the storage of lipids in tissues, such as the liver, pancreas, and target tissues for insulin, leading to cellular dysfunction and increased insulin resistance [30]. Lipo- and glucotoxicity (chronic increases in glucose and lipid concentrations that cause disrupted beta cell function) may also negatively impact the progression of insulin deficiency [31]. Increased body fat and insulin secretion (due to increased insulin resistance) may stimulate androgen production, which may further stimulate insulin secretion and consequently lead to the loss of beta cell function [4, 32-34].

Common complications related to T2DM include cardiovascular complications, diabetic neuropathy, nephropathy, diabetic retinopathy, and foot ulcers [1, 28, 29]. The relationship between T2DM and cardiovascular disease is reciprocal, with each condition acting as a risk factor for the other. Additionally, these diseases share several risk factors associated with metabolic processes [35]. Among individuals diagnosed with T2DM, the prevalence of cardiovascular disease is estimated to be 32%. Moreover, cardiovascular disease is responsible for more than half of the deaths that occurred during a 10-year period among the studied individuals with T2DM, with coronary artery disease and stroke emerging as significant contributing factors [36]. Diabetes is a leading contributor to the development of kidney disease, affecting an estimated 20–40% of individuals with diabetes. This comorbidity further elevates the risk of developing cardiovascular disease [37, 38]. Hyperglycaemia and duration of diabetes are also important risk factors for diabetic retinopathy, with an estimated prevalence of 25% among individuals with T2DM, and is the leading cause of incident blindness [39, 40]. Diabetic neuropathies are the most common complications of T2DM with an estimated 60% prevalence among individuals who have been diagnosed with T2DM for 10–15 years [41]. The prevalence of foot ulcers among individuals with T2DM is estimated to be 19–34%, which substantially elevates mortality rates, with affected individuals facing a two-and-a-half times higher risk compared to individuals with T2DM but without foot ulcers

[40, 42]. These complications underscore the significance of early identification of high-risk individuals and the implementation of preventive measures.

1.2 Biomarkers as risk factors for T2DM

Biomarkers are commonly used for the early identification of disease risk and can assist in the diagnosis, evaluation, and monitoring of specific endpoints that are either directly or indirectly associated with the biomarker [43, 44]. According to the Biomarkers Definitions Working Group [44], biomarkers objectively measure biological processes. Biomarkers are employed to measure various aspects of a medical state and are categorised into two main types of endpoints: 1) clinical endpoints provide a direct characterisation of the disease state and 2) surrogate endpoints which measure specific characteristics associated with the disease and are expected to predict clinical outcomes [45, 46]. Biomarkers may originate from different sources, including imaging, blood, and genetic biomarkers, as well as any other type of recording or survey that may be relevant to the endpoint being measured[43, 44].

While diagnostic models are used to identify and classify diseases, prognostic models are used to estimate the likely outcomes of diseases [47]. Prediction models use known information from biomarkers to obtain prognostic estimates of disease risk in the future [48]. Prediction models for assessing future risks are dependent on time intervals, particularly the duration between the measurement of a biomarker and the occurrence of the endpoint (which could manifest as the presence of disease in cases and the absence of disease in controls) [45]. Therefore, the goal of prediction models is to prevent or delay the development of potential diseases.

There are several risk prediction models for T2DM which have been validated to an extent and can be used clinically, which includes Australian Type 2 Diabetes Risk Assessment Tool (AUSDRISK) [49], Atherosclerosis Risk in Communities (ARIC) [50], Cambridge risk score [51], Finnish Diabetes Risk Score (FINDRISC) [52], The Framingham offspring study [53], the San Antonio Heart Study [54], and the Diabetes Risk Algorithm (QDiabetes) [55]. These risk scores are based on 5–10 years of follow-up and utilise either non-invasive established risk factors or a combination of non-invasive established risk factors and blood biomarkers. Several blood biomarkers are associated with an increased risk of T2DM. Despite ongoing

efforts, improvements in prediction models, particularly when compared to non-invasive established risk factors, are often marginal or even non-existent. This is because many blood biomarkers are correlated with the existing non-invasive established risk factors, which are already adequate for predicting T2DM [10, 56]. However, there is still value in exploring novel biomarkers and conducting further investigations to understand how early biomarkers can contribute to improved predictions. By enhancing the accuracy of individual predictions, we can gain a better understanding of T2DM aetiology, leading to more effective prevention and treatment strategies [57, 58].

1.2.1 Established risk factors for T2DM

Several genetic and environmental factors are associated with the development of T2DM [22]. Most prominent factors include heredity, lifestyle, and dietary habits as well as hypertension, disturbed lipid- and glucose homeostasis, and hormonal imbalance [59, 60]. Several easily obtainable, non-invasive, established risk factors for T2DM have been consistently reported in the literature. These include age, BMI, physical activity, elevated blood pressure, family history of T2DM, smoking, and waist circumference [9, 61-64]. The aforementioned prediction models, including AUSDRISK, Cambridge risk score, and FINDRISC exclusively use non-invasive clinical information such as age, sex, family history of diabetes, use of blood pressure/steroid medicine, BMI/waist circumference, physical activity, ethnicity, smoking, and diet. Extensive research has demonstrated that these models have a success rate of 74–85% in accurately discriminating between cases and controls [65]. However, these prediction models typically rely on a single baseline measurement and a follow-up period of 5–10 years. In these models, T2DM cases are usually identified based on self-reported T2DM diagnosis and/or use of diabetes medication, except the AUSDRISC study which incorporates glucose concentrations in addition to the aforementioned criteria [49, 51, 52]. Due to the length of the follow-up period and the lack of blood sample analyses confirming the absence of diabetes, the cases in these studies may have already started experiencing an acceleration of T2DM progression, thus accentuating the differences between cases and controls, while missing individuals at an earlier stage of the disease. These risk scores perform well in identifying high-risk/undiagnosed individuals; however, repeated

measurements of the same individuals with a longer follow-up period are required to better understand how risk factors change over time, encompassing all stages of T2DM progression.

1.2.2 Blood biomarkers

Several blood biomarkers related to metabolic pathways in adipose tissue, liver, and endocrine system are associated with the development of T2DM [52, 53, 66]. Currently, glucose and HbA_{1c} are used as diagnostic biomarkers for assessing glycaemic control and diagnosing diabetes (Table 1) [3, 67].

Table 1. Diagnostic criteria for prediabetes and type 2 diabetes mellitus set by the American Diabetes Association and the International Expert Committee

Prediabetes
Fasting plasma glucose: ≥7.0 mmol/l (126 mg/dl)
2-hour plasma glucose: ≥11.1 mmol/l (200 mg/dl)
HbA_{1c}: 39[a] (42[b]) – 47 mmol/mol (5.7[a] (6.0%[b]) – 6.4%)
Type 2 diabetes mellitus
Fasting plasma glucose: 5.6 – 6.9 mmol/l (100 – 125 mg/dl)
2-hour plasma glucose: 7.8 – 11.0 mmol/l (140 – 199 mg/dl)
HbA_{1c}: ≥48 mmol/mol (6.5%)
Random glucose concentration: ≥11.1 mmol/l (200mg/dl).

[a]Lower cut-off value set by the American Diabetes Association [3].
[b]Lower cut-off value set by the International Expert Committee [67].

Adipocytes are integral in the regulation of insulin secretion and sensitivity, as well as in glucose and lipid metabolism [68]. Adipocytes exert their action through the secretion of adipokines, such as adiponectin (which enhances insulin sensitivity), leptin (involved in glucose regulation and insulin sensitivity), and visfatin (exhibiting insulin-like properties) [69]. The concentrations of glucose, HbA_{1c}, total lipids, triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) reflect glucose and lipid metabolism, dysglycaemia, dyslipidaemia, as well as gluco- and lipotoxicity which are associated with T2DM [70]. Higher concentrations and unfavourable time trends of lipids, glucose, and HbA_{1c} have been observed in cases with T2DM compared to healthy controls over a 10–20-year period [71-73]. The ARIC risk score, the Framingham Offspring Study, the San Antonio Heart Study, and QDiabetes included invasive biomarkers such as HDL-C, triglycerides, fasting glucose, and HbA_{1c} in addition to non-invasive

established risk factors, which had an 80–85% chance of successfully discriminating cases and controls [65].

The presence of excess liver fats, as observed in non-alcoholic fatty liver disease (NAFLD) along with increased levels of the liver enzyme gamma-glutamyl transferase (GGT), have been observed to be associated with the development of T2DM for up to 15 years before diagnosis. These factors also share common risk factors with T2DM such as insulin resistance and individuals being overweight [71, 74-78]. Given the similarities in risk factors and the bidirectional association between NAFLD and T2DM, considering GGT levels as a blood biomarker of liver status may further reflect irregular lipid metabolism and insulin resistance that occurs during T2DM disease progression [74]. Although increased levels of GGT have consistently been associated with an increased risk of T2DM, this biomarker has not yet been assessed in a prediction model [62].

The endocrine system plays a vital role in regulating major energy metabolism processes and involves a dynamic interplay between hormones and metabolic functions, particularly in relation to insulin and glucose regulation. Dysregulation of the endocrine system may potentially affect glucose and lipid metabolism, which counteracts the secretion and action of insulin [79]. The majority of studies investigating the endocrine system have been conducted using a single baseline sample. Considering the slow progression of T2DM and strict regulation of hormones, repeated assessments of the same individuals over a longer period are required to comprehensively understand the disturbances in the endocrine system and to identify potential differences in concentrations and time trends between cases and controls.

The thyroid gland plays a crucial role in regulating cell differentiation (e.g. white and brown adipose tissues), growth, and energy metabolism [80]. Thyroid-stimulating hormone (TSH), released from the pituitary gland, regulates the production of thyroxine (T_4) and triiodothyronine (T_3) through a negative feedback mechanism. T_4 is deionised in peripheral tissues into T_3, which exhibits greater potency [80, 81]. T_3 potentially impacts glucose and lipid metabolism and substantially affects insulin sensitivity in skeletal muscles [82]. While thyroid hormones have not been incorporated into prediction models, there is evidence of an association between thyroid dysfunction and T2DM. Disruption in the thyroid function has been shown to increase the risk of developing T2DM, and individuals with T2DM often exhibit impaired thyroid function, indicating a reciprocal relationship. Notably, disturbed

insulin sensitivity is a shared characteristic in both conditions [83, 84]. Additionally, increases in thyroid hormone levels over a 5–7-year period have also been associated with an increased risk of T2DM [85, 86].

Steroid hormones are a group of hormones derived from cholesterol and include androgens and vitamin D [87]. Vitamin D is primarily involved in bone mineralisation; however, an increasing number of studies have suggested that vitamin D may potentially be associated with T2DM [88]. Sources of Vitamin D include sunlight exposure, diet, and vitamin D supplements; additionally, it is metabolised in the liver into 25-hydroxyvitamin D (25(OH)D) and subsequently into 1,25-dihydroxyvitamin D in the kidneys [89]. Vitamin D is believed to play a crucial role in glycaemic control, beta-cell function, and insulin sensitivity, which are factors associated with T2DM progression [90]. There are certain inconsistencies in the reported association between vitamin D and T2DM, with studies showing both significant and non-significant causal and observational relationships [91]. Most of the observational studies support a significant association between lower 25(OH)D levels and an increased risk of T2DM [91, 92]. A few studies conducted within a prevention program and incorporating repeated measurements and follow-up periods spanning 1.1-2.7 years, strongly support the fact that improvements in 25(OH)D status reduce the risk of T2DM [93, 94]. It has also been reported that, although not statistically significant, vitamin D supplementation in several clinical trials consistently indicates a reduced risk of T2DM [91].

Androgens play a crucial role in sexual development and reproductive health, as well as in regulating normal physiological and metabolic processes in men and women [95]. Some of the most common androgens include testosterone, androstenedione (A4), and dehydroepiandrosterone sulphate (DHEAS), which are mainly produced in the gonads and adrenals [96]. DHEAS functions as a buffer for dehydroepiandrosterone, with neither of them exerting androgenic effects independently. Instead, they serve as a precursor to active androgens, such as testosterone and A4, which then exert their action on target tissues, including the brain, reproductive organs, bone, skeletal muscles, the cardiovascular system, and adipose tissue [96]. The adrenals also produce 11-oxygenated androgens, which constitute a group of androgens that were not previously regarded as potent [97]. However, their androgenic potency has recently been recognised to play a role in several physiological processes [97]. For example, 11-ketotestosterone (11KT) has been shown to possess similar potency as testosterone [96, 97]. Moreover, 11-oxygenated androgens have exhibited stronger

associations with several diseases, such as congenital adrenal hyperplasia (CAH) and polycystic ovarian syndrome (PCOS), in comparison to classic androgens [98]. Although the association between the concentration of 11-oxygenated androgens and the risk of T2DM has not been reported, PCOS and CAH have been reported to be potentially associated with T2DM [99-101]. Thus, 11-hydroxytestosterone (11OHT), 11-hydroxyandrostenedione (11OHA4), 11-ketoandrostenedione (11KA4), 11KT, A4, DHEAS, and testosterone levels may potentially reflect the dysregulation of metabolic processes related to the development of T2DM.

There have been certain inconsistencies in the association between testosterone and T2DM; however, a meta-analysis by Ding et al., which combined data from 36 cross-sectional studies (3,825 men and 4,795 women) and seven prospective studies (368 cases), revealed a significant association between low testosterone levels in men and high testosterone levels in women with T2DM [102]. Similarly, in a retrospective cohort (70,541 men and 81,889 women) study conducted by O' Reilly et al. [103], higher testosterone levels in women and lower testosterone levels in men were significantly associated with T2DM. In a study conducted by Atlantis et al. [104], testosterone was evaluated within a prediction model for men, which incorporated both non-invasive and blood biomarkers from various validated risk scores. The findings revealed a significant association between testosterone and T2DM within the model. However, the addition of testosterone only marginally improved the discrimination between cases and controls (83% with testosterone vs. 82% without testosterone). None of the other androgens have been assessed in prediction models, whereas testosterone was only tested in men. However, the number of studies investigating 11-oxygenated androgens is notably limited. Only one cross-sectional study has reported a lack of association between 11-oxygenated androgens and T2DM, while a few studies have observed significant associations between insulin and insulin resistance [32, 105-107].

1.2.3 T2DM prevention and intervention

There are several stages of prevention, including primary, secondary, and tertiary prevention [108]. Primary prevention of T2DM involves public health measures such as education about healthier lifestyle habits and diabetes, while secondary prevention of T2DM involves the management of obese individuals and early disease detection by screening and risk prediction

to identify high-risk individuals among the general population [109]. Tertiary prevention involves disease management and the prevention of complications. Alberti et al. [9] and Sattar [56] suggested a multistep approach for the feasible implementation of risk prediction and intervention. Briefly, they suggested that simple non-invasive questionnaires should determine the initial risk and thus identify individuals at a higher risk of developing T2DM in the future. Upon identifying an individual with an increased risk, it is crucial to conduct periodic blood sample analyses to enhance risk assessment. Based on the risk level, individuals with low risk should receive general health improvement advice, while higher-risk individuals should be offered a more comprehensive intervention by health care professionals. Interventions should target lifestyle and dietary habits, and if applicable, pharmacological intervention. High-risk individuals should be offered recurring visits and risk assessments for continuous monitoring to ensure effective disease management. [9, 56]. These interventions will be effective in reducing or delaying the progression of T2DM, thus limiting the negative consequences associated with the disease, including higher mortality, lower quality of life, and increased economic burden on the health care system [110]. Currently, lifestyle counselling stands as one of the main strategies for T2DM prevention. Its aim is to promote increased physical activity, facilitate changes in dietary habits, control blood glucose levels, and prevent hyperglycaemia [110]. According to the Norwegian Directorate of Health, current risk assessment in Norway comprises a three-phase approach, as suggested by Alberti et al. and Sattar (Figure 1) [9, 56, 111]. The use of a risk assessment tool, such as FINDRISK, is recommended by primary healthcare services. Despite strong evidence supporting the effectiveness of lifestyle interventions in reducing the risk of T2DM among high-risk individuals, the Norwegian Directorate of Health considers the recommendation for a three-phased risk assessment as a low priority. This decision is based on their assessment that the current literature lacks sufficient evidence to justify the advantages outweighing the disadvantages of population-wide screening [6, 111].

After the manifestation of T2DM in an individual, the severity of the beta cell deficiency and increased insulin resistance is irreversible and may be difficult to treat [4, 5]. The first-line intervention following the diagnosis of T2DM, categorised as tertiary prevention, typically involves self-management education, counselling on lifestyle modifications, and utilisation of glucose-lowering drugs, predominantly metformin. These measures aim to reduce the severity of the disease and mitigate the risk of associated complications [112-114]. As the disease

progresses and the efficacy of initial therapy decreases, other glucose-reducing medications can be introduced in combination with metformin and lifestyle changes. Eventually, insulin administration may be required [112]. Treatment and prevention options are mainly aimed at reducing glucose concentrations and preserving beta cell mass by reducing their workload [25]. Another significant aspect of disease prevention, falling under primordial prevention strategies, involves identifying the underlying behaviours of societal circumstances that increase exposure to risk factors [115]. This approach aims to address the broader population, which can have a greater impact on the number of cases prevented compared to solely targeting high-risk individuals [116]. This means that a more targeted prevention strategy offers substantial benefits to individuals at high risk, but it does not address the underlying societal circumstances affecting the entire population. In contrast, a broader prevention strategy may have a lesser impact on individuals, but it provides significant societal benefits by reducing overall exposure to risk factors [117]. Hence, a broad prevention strategy in combination with the early identification of high-risk individuals is crucial to prevent beta cell deficiency and insulin resistance before the onset of T2DM. To achieve this, a comprehensive understanding of the early dysregulation of metabolic processes and alterations in glucose, hormone, and lipid homeostasis is required.

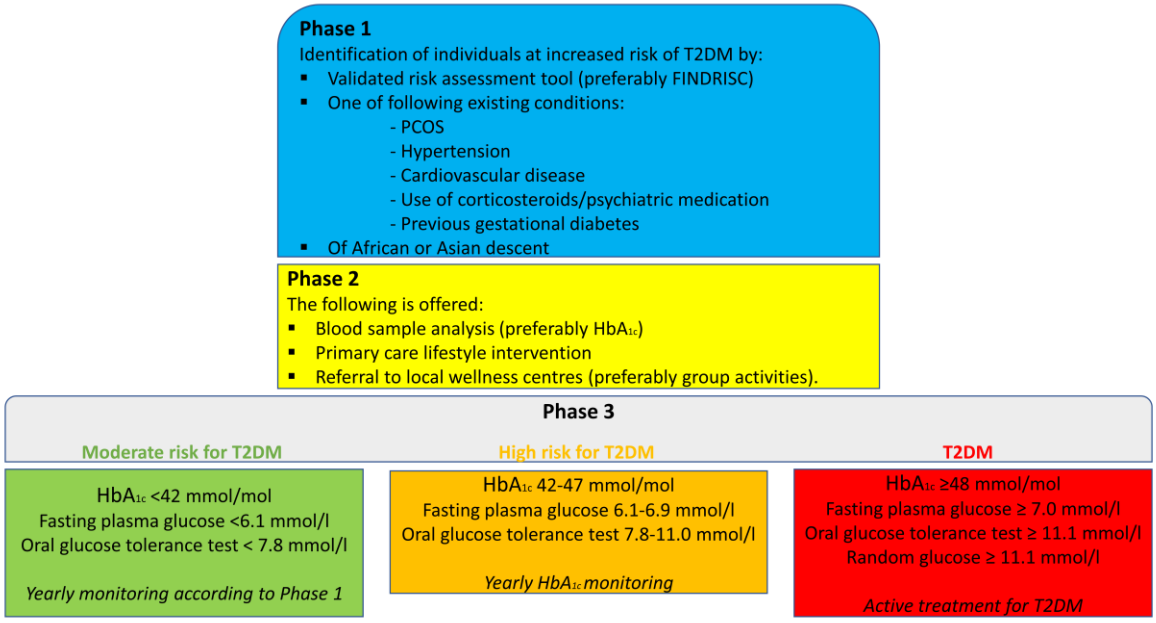

Figure 1 – Norwegian recommendation for a three-phased approach adopted from the Norwegian Directorate of Health.

1.3 Knowledge gaps addressed in this thesis

T2DM is a complex disease involving numerous potential pathways that can lead to beta-cell dysfunction and insulin resistance. The specific sequence of alterations in blood biomarker concentrations and the underlying pathophysiological mechanisms driving the development of T2DM remains poorly understood [22, 118]. Additionally, there is a notable shortage of studies that explore the associations between blood biomarkers and T2DM through repeated measurements from the same individuals over an extended period, encompassing both pre- and post-diagnosis phases. The majority of the studies predominantly utilise either cross-sectional study designs or prospective designs with a single baseline measurement. Most prospective studies have a follow-up period ranging from one to ten years, while a few studies extend up to 10–20 years [10, 62]. Very few of prospective studies have incorporated a diverse range of blood biomarkers that adequately reflect the various aspects of metabolic processes. Furthermore, there is a lack of prediction evidence for many of these biomarkers in relation to T2DM, particularly regarding their implications for the endocrine system in relation to T2DM [10]. It is evident that there is a significant gap in longitudinal observational evidence regarding the relationship between the endocrine system and T2DM [10, 62]. Further research on biomarkers for T2DM is needed to advance the understanding in this research field, with the potential to reduce the incidence of T2DM and enhance disease prognosis [119, 120]. Given the complexity and heterogeneity of T2DM, it is essential to incorporate biomarkers from multiple metabolic pathways to effectively identify and target high-risk individuals for preventive measures [120].

2 Aims of the thesis

The objective of this thesis is to investigate blood biomarkers that reflect metabolic processes linked to T2DM development and to explore the potential of these blood biomarkers as early predictors. The blood biomarkers investigated include lipids, glycaemic biomarkers, GGT, and thyroid hormones in Paper I, 25(OH)D in Paper II, and androgens (classic and 11-oxygenated) in Paper III.

Specific objectives are as follows:

1. Investigate the association between blood biomarkers and T2DM over a 30-year period.
2. Assess the pre- and post-diagnostic concentrations of blood biomarkers in T2DM cases compared with healthy controls.
3. Explore the potential of blood biomarkers to successfully enhance the predictive ability of T2DM compared to established risk factors.

3 Materials and Methods

3.1 Study population and design

The participants included in this thesis were originally enrolled in the Tromsø Study, an ongoing population-based health survey with seven surveys conducted to date (Tromsø1 – Tromsø7) [121]. The Tromsø study was initiated in 1974 in the Tromsø municipality in Northern Norway and has since gathered extensive data through questionnaires and physical examinations, ensuring repeated measurements, for a substantial portion of the participants. Blood samples were collected for various analyses and stored until further use.

This thesis used a longitudinal, nested case-control study design with up to five repeated measurements from 290 participants, including both men and women (Figure 2). The surveys used were Tromsø3 (1986/87), Tromsø4 (1994/95), Tromsø5 (2001), Tromsø6 (2007/08), and Tromsø7 (2015/16), referred to as Time-points 1 – 5 (T1 – T5). T1–T3 were pre-diagnostic time points, while T4 and T5 were post-diagnostic time points, and cases were diagnosed with T2DM after T3 and before T4. Data collected at all time points were used for Papers I and II, and data collected at the pre-diagnostic time-points T1–T3 were used for paper III. T2DM cases were recorded in the local diabetes registries and confirmed using laboratory data and medical records. The inclusion criterion for this study was that the blood samples of all the cases were available during the time-points T1–T3, which resulted in the recruitment of 76 women and 69 men. Controls were randomly selected, with an equal distribution of men and women and blood sample availability. This amounted to 290 cases and controls in each survey from T1 to T3. Out of these, 29 cases and seven control individuals were excluded from the study because they exhibited HbA_{1c} levels higher than 48 mmol/mol (6.5%) or were taking diabetes medication during the time-point T3 or earlier (in cases) and during any time point (in case of the controls). The final study sample comprised 116 cases and 138 controls at each survey from T1–T3, 57 cases and 62 controls at T4, and 50 cases and 58 controls at T5. Collectively, 989 blood samples were available for laboratory analyses. Additionally, for Paper I, participants who reported using medication for thyroid disease were excluded (11 cases and 8 controls), resulting in a total of 234 participants.

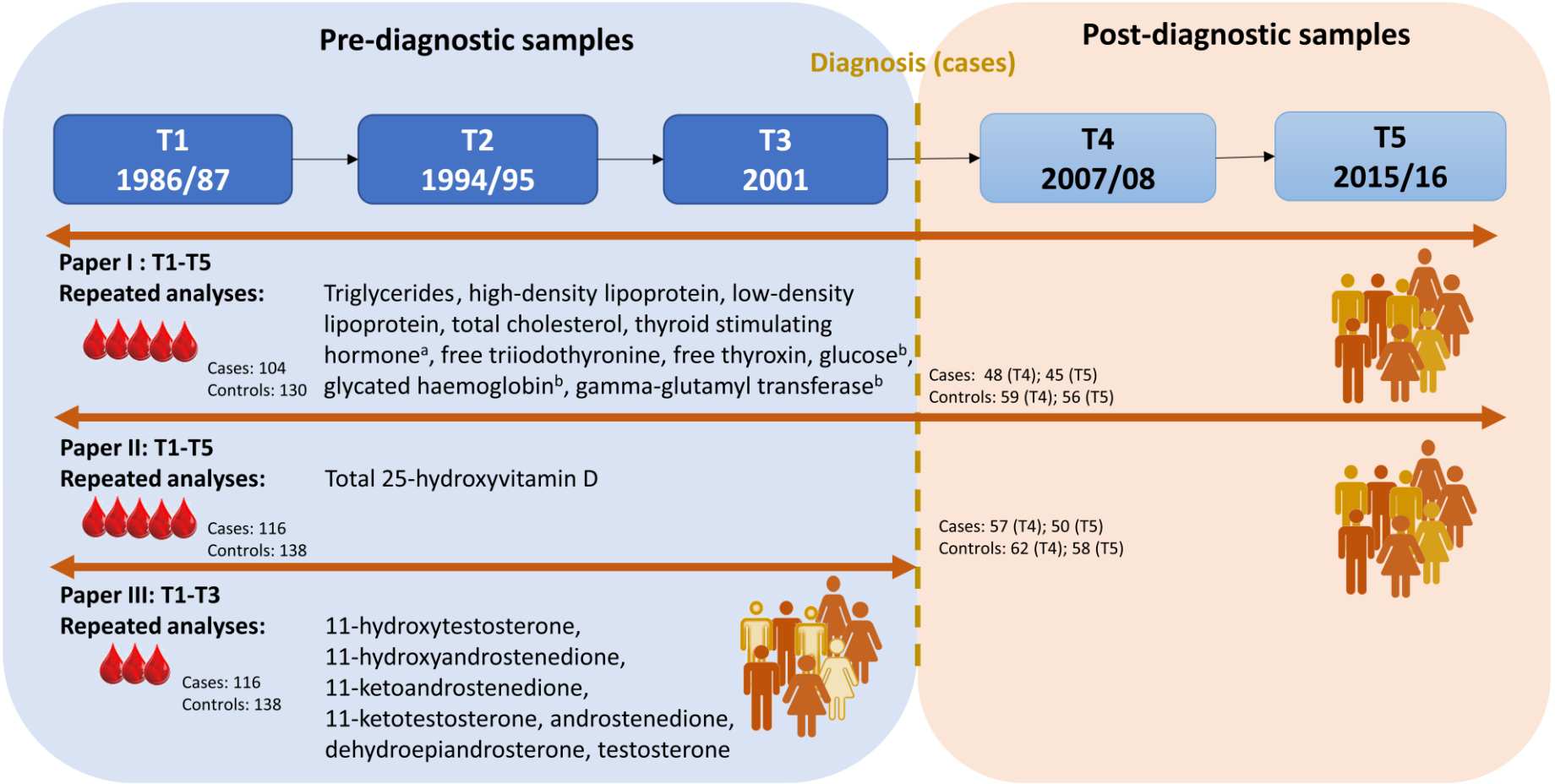

Figure 2 – Overview of the thesis displaying blood biomarkers used for all papers. The Tromsø study 1986–2016.
[a]Thyroid stimulating hormone analyses at T2 were performed at the time of blood collection.
[b]Analyses of these parameters were performed at the time of blood collection during the respective survey.
T, Time-point.

3.2 Ethical consideration

The Tromsø Study surveys collected informed consent at the time of enrolment for all participants. Informed consent was obtained from the participants at Tromsø3 (T1). For Tromsø4 – Tromsø7 (T2–T5), each participant provided a specific written declaration of consent at attendance. The Tromsø study was approved by the Regional Committee for Medical and Health Research. This thesis has been approved by the Regional Committee for Medical and Health Research for the specific study design and aims stated (reference:2015/1780/REK nord).

3.3 Data collection and laboratory analyses

Information from questionnaires, clinical examinations, and blood samples was collected during each survey [121, 122]. Information from the questionnaires used in this thesis included age; lifestyle habits (physical activity and alcohol consumption); use of medication, supplements, parity, and breastfeeding; age at menarche; menstrual status; and family history of T2DM. Information obtained from the clinical examinations included anthropometric and blood pressure measurements. Non-fasting blood samples were analysed for some biomarkers at the time of each survey; of these, GGT (at T1, T2, and T4), glucose (during T2–T5), HbA_{1c} (during T2–T5), and TSH (at T2) were included in this thesis. Additionally, serum at the time of each survey was frozen at −80ºC which was thawed in the 2020–2021 period and analysed for lipids (triglycerides, total cholesterol, HDL-C, and LDL-C), thyroid hormones (free T_3, free T_4, and TSH) for Paper I, total 25(OH)D for Paper II, as well as classic androgens (A4, DHEAS, and testosterone) and 11-oxygenated androgens (11OHA4, 11OHT, 11KA4, and 11KT) for Paper III.

For Papers I and II, all laboratory analyses were performed at the University Hospital of North Norway by the Department of Laboratory Medicine, which is accredited according to ISO 15189 standards, with coefficients of variations (CVs) ranging from <3% to <6%.

For Paper III, the analyses were performed at the Arctic University of Norway using an assay, specifically developed for this thesis. Serum samples were prepared by liquid-liquid extraction, followed by separation and detection by liquid chromatography and tandem mass

spectrometry (LC-MS/MS) (Appendix for Paper III). Six-point calibration curves and quality controls were included in each analysed batch, and the method exhibited a CV of <10%.

3.4 Statistical analyses

All statistical analyses were performed using STATA (v. 17.0, StataCorp LLC, 4905 Lakeway Drive, College Station, Texas, USA). The significance level was set at 5%, with two-sided *p* values. All the analyses were stratified according to sex. Because the study design was longitudinal with up to five time points, we decided to apply several statistical approaches to fully explore the relationship between biomarkers and T2DM, as described below. Sample characteristics were reported as means with standard deviations (SD), medians with 5 and 95 percentiles, and frequencies with percentages, with a comparison between cases and controls performed using an unpaired two-sample t-test, nonparametric test, and Pearson's χ^2 test, respectively. Further details can be found in the respective papers.

3.4.1 Covariates

The outcome of interest in this thesis was T2DM. Several covariates chosen based on the literature were included in this thesis. First, the covariates considered as established risk factors according to a consensus on T2DM risk factors [9] were age, BMI, physical activity, elevated blood pressure, and family history of T2DM. Second, the covariates considered for the relationship between 25(OH)D and T2DM identified by directed acyclic graph (DAGs) (Appendix for Paper II, Figure S1) were age, BMI, weight change between time points, physical activity, the month of blood sample collection, and cod liver oil intake. Third, the covariates considered for the relationship between androgens and T2DM, also identified by DAGs (Appendix for Paper III, Figures. S2 and S3) were age, BMI, physical activity for both men and women, use of contraceptives for women, age at menarche, menstrual status, hormone treatment replacement, and parity. Thus, in Paper I, the established risk factors were used in all multivariable models to assess associations, time trends, and predictions in relation to T2DM. In Paper II, the covariates identified by DAGs for 25(OH)D and T2DM were included in all multivariable models. In the models assessing summary variables across several time points, a month-specific 25(OH)D z-score was calculated instead of adjusting for

the month of blood sample collection. The summary variables included calculations of the area under the curve (AUC) for pre-diagnostic concentrations, as well as pre- and post-diagnostic differences in vitamin D concentrations. In Paper III, the covariates identified by DAGs for androgens and T2DM were included in all multivariable models assessing the associations and time trends of androgens in relation to T2DM, whereas, in the prediction models, the established risk factors were used instead.

3.4.2 Longitudinal data analyses

Longitudinal data analysis methods were used to explore differences in the time trends of blood biomarker concentrations between cases and controls. For Papers I and II, we used linear mixed effect models, including the respective blood biomarkers as dependent variables, with an indicator variable of time and a two-way interaction term with T2DM as well as covariates as independent variables. For these models, an unstructured variance/covariance correlation structure was chosen to account for within-group errors with a random intercept at the participant level.

For Paper III, all androgen concentrations were examined visually using histograms, as well as the Shapiro-Wilk test, and were considered to have non-normal distributions. Therefore, we used generalised estimating equations (GEE) specified with log-link and gamma distributions, which consider the non-normality of the response variables. An unstructured correlation structure for within-group correlation was applied to the GEE model.

Additionally, for this thesis, the difference (in percentage) in the mean adjusted estimated concentrations of each blood biomarker (retrieved from the adjusted longitudinal data analyses in each paper) in cases compared to the controls was plotted in line graphs to visualise the difference in all biomarkers collectively at every time point. Differences were calculated as follows: $\%\ difference = 100x\ \frac{mean(cases) - mean(controls)}{mean(controls)}$

3.4.3 Logistic regression analyses

In all studies, we employed crude logistic regression models to evaluate the association between T2DM as the outcome variable and the respective biomarkers as continuous independent variables at each pre-diagnostic time point (all time points for Paper II). The adjusted logistic regression models included the respective covariates of the three studies. In Papers I and II, similar models were constructed; however, biomarkers were used as categorical variables instead of continuous variables. In Paper II the continuous variable for 25(OH)D was measured per 5-nmol/l increments. In Paper III, the continuous variable for androgens was measured per 1- IQR (Interquartile range) increments.

3.4.4 Prediction models

The ability of blood biomarkers to predict T2DM was explored in Papers I and III at each pre-diagnostic time point. In both papers, the predictive ability was evaluated by comparing logistic regression models. One model included only established risk factors, while the other model incorporated blood biomarkers to assess their predictive capability. Blood biomarkers were chosen using a backward selection process and the best model fit was assessed using Akaike's information criterion (AIC) in Paper I and the likelihood-ratio test (LR-test) in Paper III.

Model discrimination was assessed by area under the receiver operating characteristics (AROC) to determine the level of discrimination indicated by the models. The AROC values were categorised as follows, based on the classification by Hosmer and Lemeshow: no discrimination (AROC: 0.5), poor discrimination (AROC: 0.50–0.7), acceptable discrimination (AROC: 0.7–0.8), excellent discrimination (AROC: 0.8–0.9), and outstanding discrimination (AROC: $\geq$ 0.9) [123]. A comparison between the different models and the baseline established risk factor model was assessed by improvement in AROC and determined to be significantly improved by model fit (AIC and LR-test respectively).

4 Results – Summary of papers

4.1 Study sample characteristics

During the pre-diagnostic period, 254 participants comprising 116 cases and 138 controls (47% men and 53% women) were included in this thesis. Fewer participants were included at the post-diagnostic time points, with 119 (36% men and 64% women) and 108 (42% men and 58% women) recruited at the T4 and T5 time-points, respectively. The cases and controls for both sexes were similar in age at T1 (mean difference of 2.09 and 2.50 years for men and women, respectively). Among the women, 44% were observed to be prospective cases at pre-diagnostic time points. At post-diagnostic time points, 46 and 47% of the cases were observed at T4 and T5, respectively. In men, 47% were prospective cases observed at the pre-diagnostic time points. At post-diagnostic time points, 47 and 49% of the cases were observed at T4 and T5, respectively. Cases exhibited significantly higher BMI compared to controls at all time-points, with mean differences at each time-point ranging from 2.2 to 3.3 kg/m^2 for men and 3.4 to 4.9 kg/m^2 for women. Among women, a higher percentage of cases exhibited elevated blood pressure (except at T5), a family history of T2DM (during T1–T3), and decreased physical activity (except at T1 and T3) compared to the controls. Among men, there were no significant differences between cases and controls in the proportions of elevated blood pressure, family history of T2DM, or physical activity across all time points (except at T5, where control individuals exhibited more physical activity than the cases).

4.2 Main results

The mean adjusted and estimated concentration differences (in percentages) between cases and controls for all investigated biomarkers across all time points for women and men are depicted in Figure 3 and Figure 4, respectively. In summary, women exhibited higher pre-diagnostic concentrations of free T_3, glucose, GGT, HbA_{1c}, and lipids, whereas 25(OH)D and HDL-C concentrations were lower than those in the controls (Figure 3). Furthermore, pre-diagnostic concentrations of 11-oxygenated androgens and DHEAS were often higher, while TSH tended to be lower in cases than in controls. Throughout the study period, cases exhibited a substantial decrease in total lipids, total cholesterol, and LDL-C along with a greater increase in glycaemic biomarkers, in comparison to controls. Furthermore, the cases

exhibited a substantial decrease in the pre-diagnostic concentrations of 25(OH)D, a greater post-diagnostic increase in 25(OH)D and GGT concentrations, and a post-diagnostic decrease in free T_3 concentrations. No significant differences in the changes over time were observed for the androgens.

Among men, cases exhibited higher pre-diagnostic concentrations of 11-oxygenated androgens, as well as higher pre- and post-diagnostic concentrations of 25(OH)D, glucose, and HbA_{1c} in comparison to controls, whereas pre- and post-diagnostic concentrations of TSH tended to be lower (Figure 4). Compared to controls, cases experienced a large decrease in lipid concentrations throughout the study period; a large decrease in pre-diagnostic concentrations of 25(OH)D; and an increase in post-diagnostic concentrations of 25(OH)D, GGT, and glycaemic biomarkers. No significant differences in the changes over time were observed for androgens.

Among women, positive associations were observed between decreasing concentrations of 25(OH)D and HDL-C, and increasing concentrations of free T_3, glucose, HbA_{1c}, total lipids, triglycerides, and T2DM. Among men, positive associations were observed between decreasing concentrations of testosterone and increasing concentrations of 11-oxygenated androgens, 25(OH)D, GGT, glucose, HbA_{1c}, and T2DM.

In Paper I, the models that incorporated both blood biomarkers and established risk factors exhibited higher discrimination compared to the models using established risk factors alone at all time points. The models that yielded the strongest discrimination between cases and controls included lipid, HbA_{1c}, and GGT levels in combination with BMI, physical activity, elevated blood pressure, and family history of T2DM. These models demonstrated excellent predictive ability (AROC: 0.85) in men and outstanding predictive ability in women (AROC: 0.95), seven years prior to T2DM diagnosis. Among men, the integration of 11-oxygenated androgens and testosterone with established risk factors yielded models with acceptable to excellent discrimination (AROC: 0.79-0.85) between cases and controls at T1–T3, surpassing the models that used only established risk factors with acceptable discrimination (AROC: 0.76-0.78) between cases and controls.

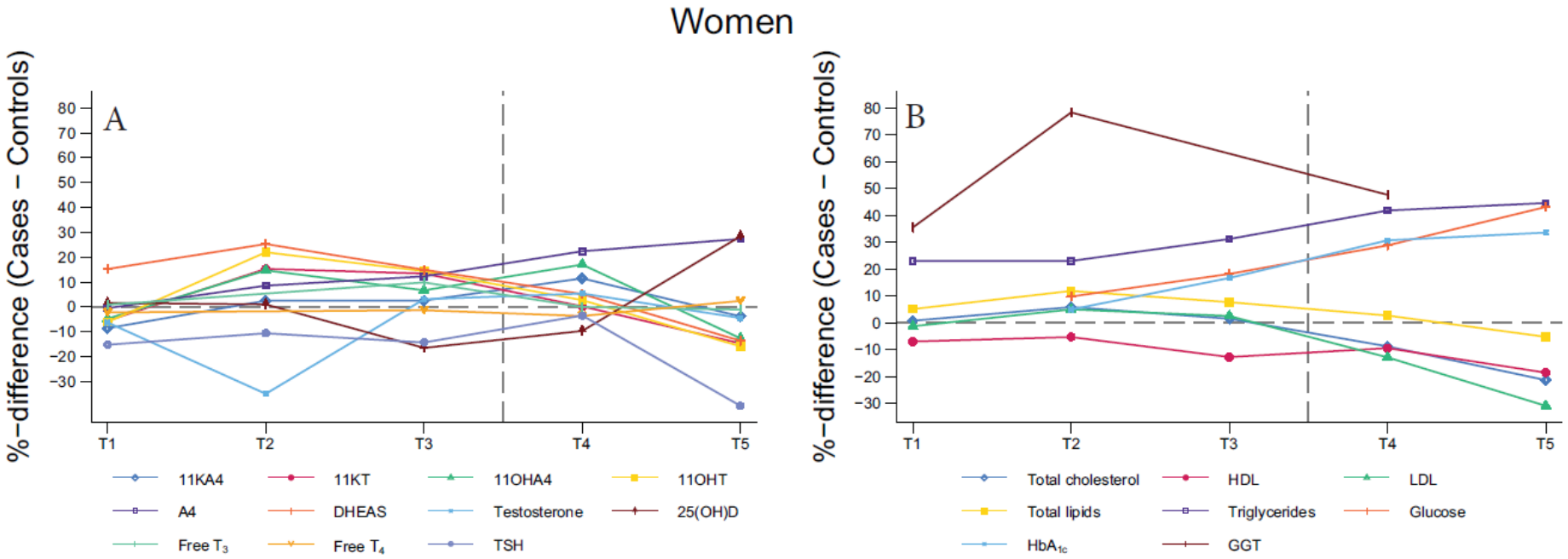

Figure 3 – Percentage difference in mean adjusted estimated concentrations of blood biomarkers in cases compared with controls at each time-point. Differences in hormones are presented in panel A; and lipids, glucose, and GGT levels in panel B. Vertical stippled lines represent the time of T2DM diagnosis in cases. Horizontal stippled lines represent 0% differences between cases and controls.

11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrosterone; 11KA4, 11-ketoandrosterone; 11KT, 11-ketotestosterone; 25(OH)D, 25-hydroxyvitamin D; A4, androsterone; DHEAS, dehydroepiandrosterone; HbA1c, Glycated haemoglobin; HDL-C, High-density lipoprotein cholesterol; GGT, Gamma-glutamyl transferase; LDL-C, Low- density lipoprotein cholesterol; Free T3, Free triiodothyronine; Free T4, Free thyroxine; TSH, Thyroid- stimulating hormone.

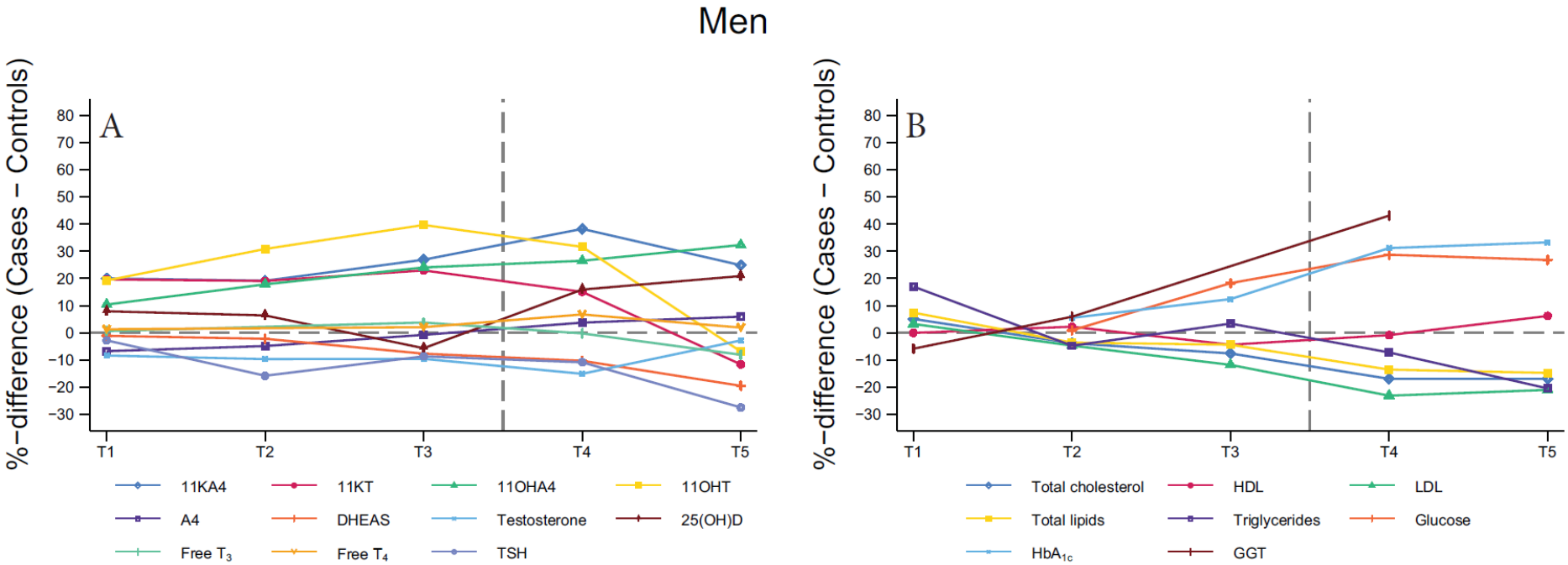

Figure 4 – Percentage difference in mean adjusted estimated concentrations of blood biomarkers in cases compared with controls at each time-point. Differences in hormones are presented in panel A; and lipids, glucose, and GGT in panel B. Vertical stippled lines represent the time of T2DM diagnosis in cases. Horizontal stippled lines represent 0% differences between cases and controls.

11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrosterone; 11KA4, 11-ketoandrosterone; 11KT, 11-ketotestosterone; 25(OH)D, 25-hydroxyvitamin D; A4, androsterone; DHEAS, dehydroepiandrosterone; HbA1c, Glycated haemoglobin; HDL-C, High-density lipoprotein cholesterol; GGT, Gamma-glutamyl transferase; LDL-C, Low- density lipoprotein cholesterol; Free T3, Free triiodothyronine; Free T4, Free thyroxine; TSH, Thyroid- stimulating hormone.

5 Discussion

5.1 Main findings

This thesis is the first of its kind to include repeated pre- and post-diagnostic measurements of a considerable number of blood biomarkers in T2DM cases and healthy controls over a 30-year period. Our results demonstrated significant differences in concentrations between cases and controls for several blood biomarkers in both men and women throughout the study period. Moreover, we observed significant associations between the concentrations of 25(OH)D, androgens, glucose, HbA1c, GGT, lipids, and thyroid hormones, and the occurrence of T2DM. These findings indicate that cases exhibit distinct lipid, glucose, and hormone homeostasis compared to controls, which may be associated with the development of T2DM. Our findings indicate that changes over time in concentrations of 25(OH)D, glycaemic biomarkers, GGT, LDL-C, total cholesterol, and total lipids differed between cases and controls. In contrast, the changes in triglycerides, HDL-C, androgens, free T_4, and TSH remained similar throughout the study period. The observed changes over time indicate a progressive worsening of blood biomarker homeostasis, which ultimately leads to the development of T2DM. Additionally, the continued deterioration of glycaemic biomarkers post-diagnosis can be attributed to beta cell dysfunction and insulin resistance, while interventions may have contributed to the improvement in the lipid profile among cases. Changes in hormones were not significantly different between cases and controls during the pre-diagnostic period, potentially due to the tightly regulated endocrine system and its feedback mechanisms. Thus, the observed differences between cases and controls might have manifested before T1 and remained relatively stable throughout the study period. Our findings highlight the reliability of using a combination of established risk factors along with lipids, GGT, and HbA_{1c} as early predictors of T2DM in both sexes, with the predictive period extending up to 15 years before diagnosis. A novel and previously unreported finding from our study is the potential association between 11-oxygenated androgens and the risk of developing T2DM. Given the significance of these results, further investigation is warranted to comprehensively understand the role of androgens in the aetiology of T2DM.

5.2 Blood biomarkers and T2DM

5.2.1 Lipid concentrations and T2DM

There were several differences in lipid concentrations between cases and controls 15 years before diagnosis. Especially reduced concentrations of HDL-C and increased concentrations of total lipids and triglycerides were positively associated with T2DM. These differences were more pronounced in women compared to men, which aligns with findings from other studies that reported stronger associations between triglycerides, HDL-C, and T2DM in women than in men. These disparities may be attributed, in part, to variations in hormone concentrations and fat deposition [124-128].

Differences in lipid concentrations between cases and controls were evident, even though there was a general decline in total lipids, total cholesterol, and LDL-C throughout the study period in both cases and controls. The accelerated decline in cases compared with controls, which started at T3, may be partly explained by the increased use of lipid-lowering drugs among the cases at T3 – T5. These findings are consistent with the results from three other longitudinal studies conducted in the general population from the Tromsø municipality, which also demonstrated improvements in lipid profiles over a period ranging from 14 to 37 years [19, 122, 129]. Hopstock et al. [122] hypothesised that the trends observed in lipid profiles could be attributed to a nationwide improvement in dietary habits during the same period covered by the current thesis. The hypothesis suggests that the reduced consumption of fatty acids and trans fats, along with the increased usage of lipid-lowering drugs, as part of a public health initiative, contributed to these changes. Studies conducted on populations from several developed countries have reported similar trends of continuous decreases in total cholesterol levels [130-135]. These authors also attributed these changes to factors such as increased awareness of the benefits of healthier lifestyles (e.g., healthier diet, increased physical activity, and reduced smoking) due to intensified public health actions as well as the increased usage of lipid-lowering drugs.

5.2.2 Glycaemic biomarkers and T2DM

As observed in both men and women, HbA_{1c} levels were higher in cases compared to controls as early as seven years before diagnosis (37 mmol/mol vs. 35 mmol/mol). Notably, this difference was significant, even though the concentrations were within the normal range for both cases and controls (20–42 mmol/mol). This finding is particularly noteworthy when compared to the diagnostic criteria for prediabetes currently employed in Norway, which is set at 42 mmol/mol by the International Expert Committee [67]. Based on these findings, it is recommended that Norway, along with other countries using similar diagnostic criteria for prediabetes, should contemplate adopting the diagnostic criteria set by the American Diabetes Association, which ranges from 39 to 47 mmol/mol. Additionally, considering a lower threshold, such as 37 mmol/mol, may be beneficial for identifying individuals at an increased risk of developing T2DM. Similarly, glucose concentrations in women were observed to be higher in cases (5.28 mmol/l) than in controls (4.68 mmol/l) at T2, seven years before diagnosis. Among men, no significant differences were observed between cases and controls in terms of glucose concentrations at T2. However, noticeable differences were evident at T3, suggesting that elevations in glucose concentrations are detectable earlier in women than in men. Similar results were reported in a systematic review by Danaei et al. [136], in which glucose concentrations in women had a stronger association with T2DM in comparison to men. The results pertaining to glycaemic biomarkers are consistent with established literature, which highlights the gradual nature of T2DM progression disease and the early detectability of disturbances in glycaemic control, even when glucose levels appear to fall within the normal range [136]. The observed post-diagnostic increases in glycaemic biomarkers aligns with expectations, as T2DM is considered an irreversible condition, highlighting the challenges in managing the disease once it develops.

Since glucose and HbA_{1c} concentrations were not available at T1, it is not possible to determine whether they were significantly associated with T2DM 15 years before diagnosis in the cases. However, considering the significant differences between cases and controls detected seven years before diagnosis, it is reasonable to assume that similar differences in glucose and HbA1c concentrations would have been observed earlier as well. This is supported by several studies examining the trajectories of glucose homeostasis and insulin resistance/sensitivity which found differences in these parameters 10–20 years before T2DM diagnosis [26, 72, 73]. Although glucose homeostasis is tightly regulated, and early insulin

resistance is compensated for by increased insulin secretion, differences can still be detected, specifically following external glucose intake [4]. This was observed in a study by Weyer et al. [27], in which T2DM cases exhibited similar fasting glucose concentrations but higher 30-minute and 2-hour glucose concentrations following an oral glucose tolerance test five years before diagnosis compared to controls. Additionally, Weyer et al. [27] observed that T2DM cases exhibited higher fasting insulin concentrations, as well as elevated insulin concentrations 30 minutes and 2 hours after the glucose tolerance test, compared to controls, highlighting the compensatory insulin secretion observed in early insulin resistance.

5.2.3 Thyroid hormones and T2DM

We observed that decreasing concentrations of free T_4 in women and increasing concentrations of free T_3 in both men and women were associated with increased risk of T2DM at T3, which aligns with observations from previous studies in which thyroid hormones are reported to be associated with insulin resistance and glucose homeostasis [84, 137]. Prospective studies by Chaker et al. [138] and Jun et al. [85] suggested that marginal differences in thyroid hormone levels within normal ranges between T2DM cases and controls were associated with an increased risk of T2DM for up to eight years before diagnosis. However, as observed in the prediction models, thyroid hormones performed poorly as early predictors of T2DM in the present thesis. The lack of measurements at T2, a smaller sample resulting from stratification by sex and time point, and the choice of a specific cut-off value in the prediction models may partly account for the limited predictive ability of thyroid hormones as early indicators of T2DM in the present thesis. The meta-analysis conducted by Rong et al. [83] indicates that the association between thyroid hormones and T2DM is multifaceted and extends beyond a simple cut-off value. Their study indicated that both hypo- and hyperthyroidism may contribute to an increased risk of T2DM. Thus, future studies are required to determine a cut-off value for both high and low concentrations of thyroid hormones for a more applicable and practical utilisation in the risk assessment of T2DM.

5.2.4 Vitamin D and T2DM

We observed that, in men, cases exhibited higher concentrations of 25(OH)D at T1 and T2 compared to controls. In both men and women, cases demonstrated lower concentrations of 25(OH)D at T3 compared to controls, which was followed by a substantial post-diagnostic increase in cases. The reason for the observed positive association between 25(OH)D concentrations and T2DM in men at T1 remains unclear, as most observational studies support the opposite relationship [92]. A possible explanation for the inconclusive observations on the association between 25(OH)D and T2DM may be the heterogeneous lifestyle and dietary habits between the study populations in the different studies, as well as variations in geographical factors leading to different levels of sun exposure. A few other studies incorporated repeated measurements of 25(OH)D concentrations; however, these studies were based on participants who received lifestyle and dietary counselling for up to 2.7 years of follow-up, which is considerably shorter than that in the present thesis [93, 94, 139]. Nevertheless, studies with repeated measurements support our findings that a pre-diagnostic decrease in 25(OH)D concentration is associated with an increased risk of T2DM. The observed differences in the changes of 25(OH)D concentrations over time between cases and controls suggest that vitamin D status may serve as an indicator of general health status. These concentrations may be influenced by factors related to disease progression, as well as potential lifestyle and/or medical interventions following diagnosis. This is supported by the fact that vitamin D may plausibly be involved in multiple physiological processes and its level is low in individuals with various diseases (such as T2DM and cardiovascular diseases). Despite the inconsistent findings from clinical trials investigating vitamin D supplementation, which do not provide conclusive evidence of risk reduction in disease outcomes, it is worth noting that vitamin D supplementation is still recommended and considered potentially important in reducing the risk of T2DM [91, 140, 141].

5.2.5 Androgens and T2DM

We observed that in men, 11-oxygenated androgens were consistently higher in cases than in controls and were thus positively associated with T2DM. Among women, 11-oxygenated androgens tended to be more prevalent in cases than in controls. In men, the post-diagnostic concentrations of 11KT and 11OHT decreased, while testosterone increased to levels similar

to those in the controls (Figure 4). To the best of our knowledge, similar results have never been reported, and studies involving 11-oxygenated androgens are scarce, particularly regarding the association between these androgens and T2DM. Therefore, there are no similar studies that can either support or contradict our results, except for one cross-sectional study by Davio et al. [105] which did not observe any significant association between 11-oxygenated androgens and T2DM. Emerging evidence from recent studies has highlighted a possible relationship between 11-oxygenated androgens, BMI, and insulin, indicating a potential association between 11-oxygenated androgens and T2DM [142]. Several enzymes involved in converting androgens are expressed in adipose tissue and upregulated by insulin [32, 33, 106, 143, 144]. Several studies have reported an association between increasing BMI, insulin resistance due to increased insulin secretion, and increased concentrations of 11-oxygenated androgens [105, 107, 145-148]. In addition, it has been proposed that the enzymes expressed in adipose tissue increase lipid synthesis which induces lipotoxicity, which in turn incites insulin resistance [143]. Thus, it is conceivable that this negative loop may result in an increased risk of T2DM [142].

In women, although the associations were not statistically significant, positive associations were observed between T2DM and higher concentrations of 11KT, 11OHA4, and 11OHT. Thus, the hypothesised explanation is plausible since cases exhibited significantly higher BMI and total lipid levels at all pre-diagnostic time points, although we did not have insulin measurements for comparison. The hypothesis put forward should be considered in light of contradictory findings. Studies have presented conflicting evidence regarding the association between 11-oxygenated androgens and insulin sensitivity or resistance, with some showing significant associations and others reporting no significant relationship [149, 150]. Notably, all previous studies were conducted on women or animals, except for those conducted by Davio et al. [105] and Schiffer et al. [147], which also included men.

5.3 Blood biomarkers as early predictors of T2DM

In this thesis, we identified several blood biomarkers which improved the discrimination between future cases and controls when assessed in prediction models with established risk factors. For instance, the earliest and strongest discrimination between cases and controls was

observed at T2 (approximately seven years before diagnosis) for GGT, HbA_{1c}, lipids, and established risk factors, with a 14–16% higher discrimination rate compared to established risk factors alone. These findings have also been observed in previous prospective studies based on only one baseline measurement; as such, we confirmed the value of these biomarkers, especially using repeated measurements for up to 15 years before diagnosis [151-153]. This indicates that the GGT, HbA_{1c}, and lipid levels as blood biomarkers are strong early predictors of T2DM and can be utilised to evaluate the future risk of T2DM.

The inclusion of blood biomarkers may pose practical challenges and require additional resources, especially when non-invasive biomarkers provide similar discriminatory capabilities, particularly when considering the time aspect, as blood biomarkers in the present thesis demonstrated greater utility at T2 and T3 in comparison to T1. To assess whether including blood biomarkers is worth the cost and effort, an impact analysis study is required. One could argue that the 4–7% increase in discrimination (for women and men, respectively) between cases and controls for the models including lipids, GGT, and established risk factors at T1 over the established risk factors model may not justify the allocation of additional resources. However, the 14–16% improvement observed at T2 is undeniably notable. Nevertheless, the inclusion of blood biomarkers in a prediction model is justified because the biomarkers used at each time point are largely the same (involving lipids, glucose measurements, and GGT) with consistent cut-off values over time. This consistency allows for added discrimination, which proves valuable in assessing the future risk of T2DM regardless of the time horizon. Therefore, adopting a multistep approach to identify individuals at risk of T2DM is logical, regardless of the time-points. This approach involves using a simple non-invasive risk score and subsequently applying a more comprehensive risk model that includes blood biomarkers for high-risk individuals. Numerous studies and meta-analyses support the effectiveness of primary and secondary prevention programmes, particularly for long-term prevention, in reducing the risk of T2DM and are considered cost-effective when compared to the treatment of diagnosed T2DM [6, 154-156]. Even at the national level, implementing primordial prevention strategies that target underlying risk factors, such as a sedentary lifestyle and obesity, is suggested as a means to reduce the risk of T2DM by encouraging individuals to adopt a more physically active and healthier dietary lifestyle [154].

5.4 Clinical relevance

In both men and women, the regular analysis of blood biomarkers such as lipids, glucose/HbA_{1c}, and GGT, in combination with established non-invasive risk factors, effectively identifies individuals at high risk of T2DM for at least seven years before diagnosis. As a result, the inclusion of additional blood biomarkers may be considered redundant and cost inefficient. In men, 11-oxygenated androgens and testosterone have shown promising results as potential risk factors for T2DM and may be included in risk assessments to better identify high-risk individuals. However, 11-oxygenated androgens and testosterone yielded at best 5% higher discrimination between cases and controls, except for T3, where testosterone and 11OHT together increased discrimination by 9% compared to the established risk factors. As 11-oxygenated androgens are not routinely analysed, this may negate the benefits of a simple and strong prediction model provided by risk factors such as lipids, glucose/HbA_{1c}, and GGT.

Special emphasis should be placed on the contribution of HbA_{1c} to the improvement in prediction abilities. Inclusion of HbA_{1c} along with established non-invasive risk factors, in a prediction model, may provide a simple and effective approach for identifying individuals at high risk of T2DM. However, this simplified model may result in a lower discrimination rate compared to models that incorporate additional blood biomarkers. Multiple studies and proposals suggest that HbA_{1c} could be comparable to or potentially superior to plasma glucose as an early predictor of T2DM, particularly when utilised in combination with non-invasive risk factors [151, 157, 158]. This approach could potentially reduce the number of undiagnosed individuals as HbA_{1c} also acts as a diagnostic biomarker for T2DM. As mentioned earlier, the Norwegian Directorate of Health has put forth a recommendation for a three-staged approach to risk prediction and intervention; however, it is important to acknowledge that this recommendation holds a low priority. Our results emphasise the significance of increasing the priority of those recommendations within primary health care settings. Early risk prediction and intervention can have significant benefits in reducing the risk of T2DM and its associated complications. This not only enhances the quality of life for individuals but also alleviates the health care burden and potentially reduces the economic burden [6, 154-156]. Roberts et al. [6] concluded that there is significant evidence of T2DM risk reduction among high-risk individuals through individual prevention and intervention

programs. They also observed that prevention programs prove to be cost-effective, particularly when appropriate resources are allocated [6].

6 Methodological considerations

The participants in this thesis were selected based on an ongoing population-based health survey called the Tromsø Study. This thesis adopts a longitudinal study design with repeated measurements, encompassing multiple surveys that track the same individuals over time. Such an approach allows for the investigation of changes over time and the evaluation of the predictive ability of biomarkers [159]. A cross-sectional study compares several response variables measured at a single time-point between two different groups of interest, thus achieving between-individual comparisons, but it loses information on within-subject changes such as growth, lifestyle changes, and aging [160]. The longitudinal design adopted in this thesis provides additional information on within-subject changes, allowing each participant to act as their control from one time point to another [159]. Cross-sectional studies, as opposed to longitudinal studies, do not allow the accurate time-sequencing of events [161]. Thus, longitudinal study designs are superior in assessing time trends for individuals, as well as in aetiological research, compared to serial cross-sectional study designs, which are not specific to individuals [161].

In addition to a longitudinal design, this thesis incorporated a nested case-control design. All men and women who exhibited T2DM diagnosis after a specific time point and who had attended and donated blood samples in three repeated surveys before diagnosis and possibly two samples after diagnosis were ascertained as cases, with an equal number of controls. The availability of a biobank greatly enhances the suitability of this study design for examining biomarkers and assessing a specific outcome It offers cost and time efficiencies in data collection while retaining statistical efficiency. Additionally, the inclusion of repeated measurements helps to mitigate individual differences between time points [161, 162]. This allowed us to analyse various blood biomarkers feasibly while achieving a strongly balanced dataset during the pre-diagnostic period. However, it is important to acknowledge a limitation of this approach regarding the investigation of the longitudinal association between biomarkers and outcomes. Since all cases were diagnosed after the same time point, it posed certain challenges in directly assessing the longitudinal association between biomarkers and outcomes. Investigation of the temporal patterns of the biomarkers was assessed using linear mixed models or generalised estimating equations; however, the associations had to be

assessed cross-sectionally. This was because the time-point of diagnosis (T3–T4) did not vary between the cases.

Missing data are a source of bias which may also falsely increase variance [48]. We had complete data available on blood biomarkers, except for GGT at T3 and T5, glucose, and HbA_{1c} at T1, and free T_3 and T_4 at T2. Furthermore, we possessed only partial data on glucose, HbA_{1c}, and TSH at T2; HbA_{1c} at T3; and GGT at T4. These blood biomarkers were missing owing to the lack of analyses conducted at the time of blood sample collection, and insufficient serum available for thyroid hormone measurements at T2. Nevertheless, we are confident that these missing biomarkers do not introduce any biases in our findings. However, owing to the loss of information caused by the lack of measurements, we cannot exclude the possibility that the results and conclusions of this thesis may have been affected. The linear mixed effect and GEE models were approached with an "available data" method, which meant that all available observations were used in the analysis, and for the logistic regression models, a "complete case" method approach was applied which meant that subjects with missing values on any variables included in the models were excluded [159].

6.1 Biases

Potential biases, including selection bias, information bias, and confounding bias, might compromise the validity of the study and yield misleading results and conclusions if left unchecked [163]. These biases may occur at each stage of a research project, such as study design, data collection, analysis, interpretation, and reporting of results [164].

6.1.1 Information bias

Information bias refers to errors that may occur when measuring the exposure and outcome of interest, where the gathered data may be incorrectly measured or classified [165]. This may occur because of errors in laboratory measurements, questionnaires, outcome identification (T2DM), or any part of the data collection process [161, 163]. This was accounted for in the present thesis since all examinations and blood sampling were performed at a single hospital, with the same clinical equipment and questionnaires for all participants. Additionally, the

enrolled participants were from a wide age range and repeated measurements from the same population were conducted [121]. However, measurement errors cannot be ruled out, although in this study laboratory analyses have documented the accuracy and precision of analytical performance, and were controlled by quality controls to ensure that the results did not deviate from the expected values [166]. T2DM was identified by local diabetes registries and strengthened by available blood samples (HbA_{1c}). Biases arising from the use of questionnaires are inherent in observational studies, as participants may incorrectly recall certain events or even misjudge them (knowingly or unknowingly) [165]. For instance, we cannot exclude the possibility that the participants exaggerated or underestimated physical activity, alcohol intake, use of medicines/supplements, or even a family history of T2DM. Although the measurements of blood biomarkers are objective and; thus, not subject to recall bias, biases based on measurement errors are alleviated by the use of quality controls and continuous calibration control of analytical instruments [164]. Biases that may have arisen from the questionnaires may have also been partly amended using repeated measurements for all participants, as the questionnaires between surveys aided in the correction of inconclusiveness in most variables.

6.1.2 Selection bias

Selection bias may occur when study participants are not comparable in other aspects aside from outcome or exposure of interest [165]. Several sources may cause selection bias, including the fact that attenders and non-attenders (and participants who withdraw from the study) might have different characteristics (for example, lifestyle habits, absence/presence of conditions, sex, and age), or that the location where the study enrolled its participants is not comparable (for example, hospitalised participants or cases and controls being chosen from different settings) [161, 163]. In the Tromsø study, it was observed that non-attenders tended to be younger, had a higher proportion of being unmarried and a higher percentage tended to be men [121]. Specifically in Tromsø2, which is an earlier survey from 1980, and not included in this thesis, a higher percentage of non-attenders were identified as daily smokers or ex-smokers [167].

The inclusion criteria for this thesis specified the outcome, which required all cases to be diagnosed with T2DM after T3 and to have available blood samples at three time-points prior

to the diagnosis. Based on these criteria, some of the cases also had available blood samples at T4 and/or T5. All controls were selected at random if they were free of T2DM throughout the study period, with an equal number of men and women as the cases, ensuring that they had attended the same surveys with blood samples available at the same time-points as the cases. In the Tromsø Study surveys included in this thesis, the general population was invited regardless of exposures and health outcomes, and they were all residents of the Tromsø municipality. This reduces selection bias because the study population is a good representation of the general population; however, it also limits generalisability because of the lack of ethnic diversity. Nevertheless, the invitation to participate was voluntary; thus, biases may arise owing to differences between attenders and non-attenders, which are inherent in observational studies [163].

6.1.3 Confounding

For an appropriate assessment of the association between the measured exposure and outcome, several potential confounders need to be considered to account for the inherent differences between cases and controls. A confounder is a variable that is associated with but not caused by the measured exposure and is a risk factor for the outcome, thus distorting the association [168]. Failure to control for confounders increases the risk of misinterpreting the associations studied, resulting in false conclusions. Several confounders were considered in this thesis to properly interpret the measured exposures. The established risk factors for T2DM along with potential confounders identified by DAGs and the existing literature for the measured exposures, were adjusted for. However, since we did not have access to all the collected data, such as detailed dietary intake, unavailability of data for several biomarkers of inflammation, and potentially unknown confounders, we could not determine their influence on the results. Although we attempted to adjust for confounders, the aim was not to establish causality but rather to study the exposures of interest and their associations with T2DM by adjusting for the influences of known risk factors and potential confounders. However, although we attempted to control for several confounders, we cannot exclude the possibility of unmeasured confounders or residual confounders arising from measurement errors. This study aimed to explore the differences at each time-point, time trends, longitudinal associations, and predictive ability of blood biomarkers, particularly in relation to established

risk factors and known factors that influence concentrations, such as sun exposure and 25(OH)D concentrations. The use of blood biomarkers as surrogate endpoints in risk assessments serves the purpose of predicting clinical outcomes based on existing scientific evidence, including an understanding of pathophysiological processes [44]. According to the criteria for considering a biomarker as a surrogate endpoint, it is not necessarily for the biomarker to be causally linked to the outcome; instead, it should possess sufficient robustness to accurately predict the outcome [46].

6.2 Validation

AROC alone is not an adequate measure of discrimination, and other methods for validating prediction models are required to avoid overfitted models and/or overly optimistic results. Examples of validating prediction models include performing calibrations (such as testing the models for goodness of fit), internal validation (such as splitting the study population into two sets, with one being a model development set and the second a validation set), and external validation (testing the model from the population it was developed in a new population) [169, 170]. External validation concerns whether the results from the studied population can be generalised to other populations [163]. Most often, the model will perform optimally in the data set it was developed in because of overfitting, which occurs when predictors are incorrectly contrived and usually results in higher risk estimates and discrimination than what would be observed in the general population [169]. As such, the original model developed in a specific study population needs to be tested, unaltered, in a different population, and reassessed for predictive ability [170]. In this thesis, we tested the performance of the models by testing their goodness of fit by applying AIC in Paper I and LR-test in Paper III to reduce the impact of overfitting. To ensure the credibility and generalisability of our results, it is imperative to conduct external validation using different study populations. Although our findings may seem optimistic within our dataset, testing them in other contexts will enhance their credibility and applicability in clinical practice [170]. External validation is also required to verify generalisability, as the results are based on participants from the Tromsø municipality, and lifestyle habits, genetic variation, and environmental variation may differ from those of other populations and geographical regions.

7 Concluding remarks

This thesis provides compelling evidence of noticeable differences in the biomarkers associated with metabolic processes among future T2DM cases well in advance of their diagnosis. We observed adverse time trends for lipids, GGT, 25(OH)D, and glycaemic biomarkers before diagnosis, which subsided or improved after diagnosis, except for glycaemic biomarkers. Although non-invasive established risk factors provided acceptable to excellent predictive abilities, the addition of GGT, HbA_{1c}, and lipids significantly improved the prediction models. The clinical significance of including blood biomarkers might be questioned since non-invasive biomarkers have been shown to have acceptable discrimination; however, blood biomarkers may improve the model to achieve excellent discrimination. Thus, it seems justifiable to include GGT, HbA_{1c}, and lipids after initial screening by non-invasive risk factors to achieve a more robust risk assessment. These results need to be externally validated, particularly the 11-oxygenated androgens require further study to prove their clinical significance, as there are currently no reference ranges or cut-off values, and these androgens are not routinely analysed. The positive outcomes observed from nationwide programs aimed at reducing lipid levels in the general population provide encouraging evidence for the potential success of targeted intervention programs for T2DM. By combining these targeted interventions with broader policy changes promoting a healthier lifestyle, it is feasible to achieve a reduction in glucose levels and a decline in the incidence of T2DM. It is crucial to not only identify behaviours and circumstances that increase the risk of T2DM but also to identify high-risk individuals to implement interventions for disease prevention. Regardless of the specific risk assessment tool used, this comprehensive approach is essential.

8 Future perspective

As the predictors in the models presented were determined for the specific subjects included in this thesis, the predictive ability was optimal for the data used here [57]. To establish a proper predictive performance, models should be externally validated in different populations [170]. The findings of this thesis highlight the potential of androgens, particularly the 11-oxygenated androgens, as potential biomarkers for evaluating the risk of T2DM. However, further research is needed to establish a more robust understanding of the relationship between androgens and the pathophysiological process involved in T2DM. Future research should aim to investigate potential causal links and provide a deeper understanding of the underlying mechanisms. More observational studies are needed to address the association between 11-oxygenated androgens and T2DM since this association is severely understudied. The emerging relationship between 11-oxygenated androgens and other diseases, such as PCOS, coupled with the potential relationship with T2DM, highlights the importance of considering routine analyses of 11-oxygenated androgens in clinical practice. Future studies should assess both hypo- and hyperthyroidism, as well as differences within the normal range, to elucidate the relationship between thyroid hormones and T2DM. Lipids, glycaemic biomarkers, and GGT appear to be promising predictors for the early risk assessment of T2DM; however, the cost-effectiveness and feasibility of implementing these biomarkers should be evaluated to determine whether the additional cost and effort of incorporating them into risk assessment models is justified compared to just using a simple risk score with non-invasive biomarkers.

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Paper I

Allaoui G, Rylander C, Averina M, Wilsgaard T, Fuskevåg OM, Berg V

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RESEARCH ARTICLE

Longitudinal changes in blood biomarkers and their ability to predict type 2 diabetes mellitus—The Tromsø study

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Abstract

Introduction: Identification of individuals at high risk of developing type 2 diabetes mellitus (T2DM) is important for early prevention of the disease. Once T2DM is established, it is difficult to treat and is associated with cardiovascular complications and increased mortality. We aimed to describe pre- and post-diagnostic changes in blood biomarker concentrations over 30 years in individuals with and without T2DM, and to determine the predictive potential of pre-diagnostic blood biomarkers.

Methods: This nested case–control study included 234 participants in the Tromsø Study who gave blood samples at five time points between 1986 and 2016: 130 did not develop T2DM and were used as controls; 104 developed T2DM after the third time point and were included as cases. After stratifying by sex, we investigated changes in pre- and post-diagnostic concentrations of lipids, thyroid hormones, HbA_{1c}, glucose and gamma-glutamyltransferase (GGT) using linear mixed models. We used logistic regression models and area under the receiver operating characteristic curve (AROC) to assess associations between blood biomarker concentrations and T2DM, as well as the predictive ability of blood biomarkers.

Results: Cases and controls experienced different longitudinal changes in lipids, free T_3, HbA_{1c}, glucose, and GGT. The combination of selected blood biomarker concentrations and basic clinical information displayed excellent (AROC 0.78–0.95) predictive ability at all pre-diagnostic time points. A prediction model that included HDL (for women), HbA_{1c}, GGT, and basic clinical information demonstrated the strongest discrimination 7 years before diagnosis (AROC 0.95 for women, 0.85 for men).

Conclusion: There were clear differences in blood biomarker concentrations between cases and controls throughout the study, and several blood biomarkers were associated with T2DM. Selected blood biomarkers (lipids, HbA_{1c}, GGT) in combination with BMI, physical activity, elevated blood pressure, and family history of T2DM had excellent predictive ability 1–7 years before T2DM diagnosis and acceptable predictive ability up to 15 years before diagnosis.

KEYWORDS
biomarkers, blood test, health service, longitudinal survey, preventive, risk factors, type 2 diabetes mellitus

1 | INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) has increased substantially over the past few decades and is one of the most important global health challenges of the 20th century.[1] The disease is characterized by insufficient insulin secretion and/or insulin resistance and established risk factors include among other obesity, sedentary lifestyle, excess dietary intake, and genetic factors.[2] Previous longitudinal studies of repeated pre-diagnostic measurements have demonstrated increases in lipid and glucose concentrations 1.5–20 years before T2DM diagnosis, with steeper increases closer to diagnosis.[3–10] Thus, disruption of metabolic homeostasis involving lipids, thyroid hormones, glucose, and liver enzymes is associated with T2DM.[5,8,9,11–13] However, the sequence of this disruption and its relative contribution to the progression from normal to impaired glucose tolerance, and ultimately to T2DM, remains unknown.[14,15]

Prediabetes (i.e., higher-than-normal blood glucose concentrations) precedes T2DM. Once T2DM has manifested, it is irreversible, difficult to treat, and associated with cardiovascular complications and increased mortality.[16–18] The identification of blood biomarkers and the development of risk score models for prediabetes and T2DM are therefore highly relevant, as they will enable early identification of high-risk individuals. There are currently many risk score models for diabetes (reviewed by Buijsse et al.[19]) most are based on basic clinical information like age, body mass index (BMI), physical activity, blood pressure and genetic predisposition, but some also include blood biomarkers. For instance, the FINDRISC (including basic clinical information as well as daily consumption of vegetables, fruits or berries, and history of high glucose) and the Framingham (including basic clinical information as well as high-density lipoprotein (HDL) and triglycerides) risk scores for diabetes have been shown to successfully identify high-risk individuals 5–7 years before diagnosis.[20,21]

Several studies of risk score models have shown that adding blood biomarkers to basic clinical information improves predictive ability,[4,20,22] especially biomarkers involved in glycaemic processes, uric acid, and lipids. However, most studies on prediction models are based on a single baseline blood sample.[4,23] The Tromsø Study contains blood biomarker concentrations and basic clinical information for up to five time points. Hence, we aimed to describe pre- and post-diagnostic changes in blood biomarker concentrations over 30 years in individuals with and without T2DM, and to determine the predictive potential of pre-diagnostic blood biomarkers.

2 | METHODS

2.1 | Study population

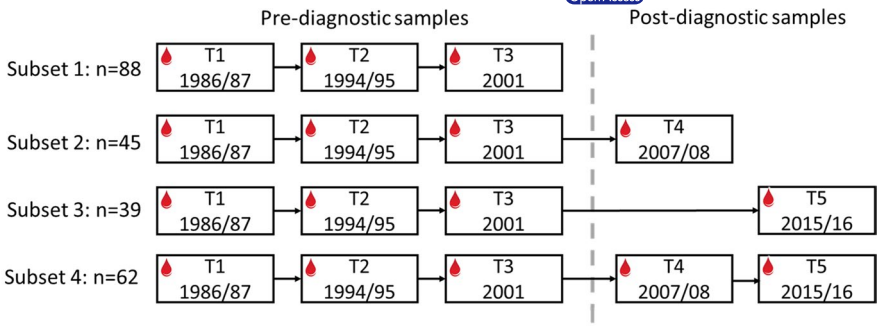
The Tromsø Study is a population-based health survey carried out in the Tromsø municipality in Northern Norway. The first survey, Tromsø1, was carried out in 1974, and six more surveys followed (Tromsø2-Tromsø7), one about every 6–7 years. During each survey, participants completed questionnaires, underwent a clinical examination and gave a blood sample.[24,25]

The present, longitudinal, nested case–control study includes blood samples collected from the same individuals at five time points: Tromsø3 (1986/87), Tromsø4 (1994/95), Tromsø5 (2001), Tromsø6 (2007/08) and Tromsø7 (2015/16). Hereafter, Tromsø3-Tromsø7 will be referred to as time point 1–5 (T1–T5), where cases developed T2DM after T3. Hence, T1–T3 was defined as the pre-diagnostic time period, whereas T4 and T5 were defined as the post-diagnostic time period.

Initially, all participants with a T2DM diagnosis were recorded in a local diabetes registry between 2000 (T3) and 2006 (T4), and available pre-diagnostic serum samples were eligible for inclusion as cases (76 women, 69 men). We then randomly selected 76 women and 69 men who participated in the same surveys, had serum samples for T1–T3 and had no T2DM diagnosis recorded in a local diabetes registry during the surveys as controls. Of the initial 290 participants, we excluded 29 cases with glycated haemoglobin (HbA_{1c}) ≥48 mmol/mol (6.5%) before or at T3, and seven controls with HbA_{1c} ≥48 mmol/mol (6.5%) at any time point. We also excluded participants who reported using medications that could affect glucose and thyroid hormone concentrations before T3 (8 controls, 2 cases). Thus, the final study population comprised 234 individuals (104 cases, 130 controls). Of these, 88 had blood samples for T1–T3 (38 cases, 50 controls), 45 (21 cases, 24 controls) had samples for T1–T4, 39 (18 cases, 21 controls) for T1–T3 and T5, and 62 (27 cases, 35 controls) had blood samples for T1–T5 (Figure 1). All participants gave informed consent at the time of each survey. The study protocol was approved by the Regional Ethics Committee, REK, nord (REK reference: 2015/1780/REK nord).

2.2 | Questionnaires, clinical examination and blood collection

The Tromsø Study questionnaire and measurements have been described in detail elsewhere.[24,25] Briefly, each survey included a



Blood biomarkers	(n)				
Triglycerides	234	232	234	107	101
Total cholesterol	234	232	234	107	101
HDL	234	232	234	107	101
LDL	234	232	234	107	101
Free T_3	234	NA	234	107	101
Free T_4	233	NA	234	107	101
TSH	234	89[a]	234	107	101
HbA_{1c}[b]	NA	184	201	168	110
Glucose[b]	NA	196	234	169	113
GGT[b]	233	234	NA	92	NA

[a]TSH analyses were performed at time of blood collection in 1994/1995.
[b]All analyses were performed at the time of blood collection at the respective survey.

FIGURE 1 Study flow chart presents the study sample according to participation in three or more surveys, and how many blood samples were analysed for the different biomarkers at each time point (T1–T5). HbA_{1c}, Glycated haemoglobin; HDL, High-density lipoprotein; GGT, Gamma-glutamyltransferase; LDL, Low-density lipoprotein; NA, not available; T, Time point; T_3, Triiodothyronine; T_4, Thyroxine; T2DM, type 2 diabetes mellitus; TSH, Thyroid-stimulating hormone. The Tromsø Study 1986–2016

questionnaire that collected information on lifestyle habits, self-reported diseases such as diabetes, family history of diseases including T2DM, parity and breastfeeding. A clinical examination was also conducted at each survey and included measurements of weight, height, waist circumference and blood pressure, among others, and the collection of non-fasting blood samples. Several analyses were performed in fresh blood samples; serum samples were frozen and stored for later use.[25]

2.3 | Laboratory analyses and availability of blood biomarkers

Serum samples were thawed and analysed for triglycerides, total cholesterol, low-density lipoprotein (LDL), HDL, free triiodothyronine (T_3), free thyroxine (T_4) and thyroid-stimulating hormone (TSH), but serum samples from T2 were insufficient for analyses of free T_3, free T_4 and TSH. Data from previous analyses carried out at the time of blood collection were available for TSH (T2), HbA_{1c} (T2–T5), glucose (T2–T5) and gamma-glutamyltransferase (GGT; T1-T2, T4). Included blood biomarkers varied at each time point (Figure 1). All analyses were performed at the University Hospital of North Norway, Department of Laboratory Medicine, using routine, established procedures. Serum concentrations of triglycerides, total cholesterol, LDL, HDL, free T_3, free T_4, TSH, glucose and GGT were determined using the Cobas® 8000 platform (Roche Diagnostics, Switzerland). Until 2006, GGT was analysed at 37°C in a Hitachi 737 Automatic Analyser using commercial kits (Boehringer Manheim, Germany) according to the recommendations of the Scandinavian Enzymes Committee.[26] HbA_{1c} was determined by high-performance liquid chromatography using an automated analyser (Variant II, Bio-Rad Laboratories). Laboratory personnel were blinded to the sample order and survey number. The laboratory is certified according to the ISO 151189 standard.[27] Quality controls are run routinely, at three different concentrations every day, and the laboratory also participates in the external quality assessment program, Lab Quality.[28] Total lipids (g/L) were calculated according to the formula[29]:

$$\text{Total lipids} = 2.27 \times \text{total cholesterol} + \text{triglycerides} + 0.623$$

2.4 | Statistical analyses

Blood biomarker concentrations and demographic variables are reported as means with standard deviations, medians with 5 and 95 percentiles, and/or frequencies with percentages. Sample characteristics were compared between cases and controls at each time point using unpaired two-sample t-tests for continuous variables and Pearson's chi-squared for categorical variables.

Linear mixed effects models were used to explore the rate and significance of changes in blood biomarker concentrations at T1–T5, between and within cases and controls, after adjusting for the following established risk factors for T2DM[30]: age (continuous), BMI (continuous), physical activity (active: ≥3 h/week of light activity and/or ≥1 h hard exercise/week or sedentary: <3 h/week of activity that provoked transpiration or no activity), elevated blood pressure (systolic blood pressure ≥130, diastolic blood pressure ≥85, and/or if the subject was taking blood pressure medication, yes/no) and family history of T2DM (siblings and/or parents with T2DM, yes/no). Blood biomarkers were used as dependent variables (continuous), whereas T2DM status, established risk factors and indicator variables of time with interaction terms with T2DM status were used as independent

TABLE 1 Characteristics of the study sample across five surveys of the Tromsø Study 1986–2016

			Pre-diagnostic time points			
			T1 (1986/87)		T2 (1994/95)	
			Mean (SD)	ΔMean case-control (95% CI)	Mean (SD)	ΔMean case-control (95% CI)
Age (years)	Women[a]	Case	45.3 (6.31)	1.46 (−1.48, 4.39)	53.3 (6.31)	1.46 (−1.48, 4.39)
		Control	43.9 (8.98)		51.9 (8.98)	
	Men[b]	Case	48.4 (8.61)	2.05 (−1.57, 5.66)	56.4 (8.61)	2.05 (−1.57, 5.66)
		Control	46.4 (10.7)		54.4 (10.7)	
Parity (*n*)	Women[a]	Case	2.66 (1.56)	0.24 (−0.35, 0.83)	2.85 (1.38)	0.34 (−0.21, 0.89)
		Control	2.42 (1.56)		2.51 (1.45)	
Breastfeeding (months)	Women[a]	Case	NA	NA	13.5 (11.5)	−1.52 (−6.22, 3.19)
		Control	NA	NA	15.0 (10.8)	
Weight (kg)	Women[a]	Case	71.9 (12.1)	8.41 (4.39, 12.4)***	77.5 (13.8)	10.7 (6.06, 15.4)***
		Control	63.4 (10.0)		66.7 (11.8)	
	Men[b]	Case	85.1 (12.9)	6.94 (2.81, 11.1)**	88.5 (13.3)	7.41 (3.07, 11.8)**
		Control	78.2 (9.24)		81.1 (10.2)	
BMI (kg/m^2)	Women[a]	Case	27.5 (4.38)	3.63 (2.14, 5.13)***	29.8 (5.13)	4.54 (2.74, 6.34)***
		Control	23.9 (3.83)		25.3 (4.72)	
	Men[b]	Case	27.5 (3.55)	2.94 (1.77, 4.10)***	28.6 (3.49)	3.03 (1.83, 4.24)***
		Control	24.6 (2.73)		25.6 (3.03)	
Waist circumference (cm)	Women[a]	Case	NA	NA	93.0 (11.4)	11.8 (6.39, 17.3)***
		Control	NA	NA	81.2 (9.95)	
	Men[b]	Case	NA	NA	101 (7.89)	7.28 (4.19, 10.4)***
		Control	NA	NA	93.7 (7.37)	
Diastolic blood pressure (mmHg)	Women[a]	Case	81.3 (10.3)	6.14 (2.31, 9.97)**	83.9 (12.2)	4.93 (0.53, 9.33)*
		Control	75.1 (10.5)		79.0 (11.8)	1.90 (−2.21, 6.01)
	Men[b]	Case	85.6 (9.67)	3.36 (−0.32, 7.04)	85.7 (11.2)	
		Control	82.2 (10.2)		83.8 (11.1)	
Systolic blood pressure (mmHg)	Women[a]	Case	131 (16.0)	7.49 (1.54, 13.4)*	142 (20.0)	7.36 (−0.09, 14.8)
		Control	124 (16.3)		135 (20.5)	
	Men[b]	Case	139 (14.2)	3.84 (−1.75, 9.43)	146 (19.8)	6.18 (−0.69, 13.0)
		Control	135 (15.9)		139 (17.4)	

Abbreviations: BMI, body mass index; T, time point.

[a]Fifty cases and 69 controls at T1–T3, 44 cases and 53 controls at T4, 26 cases and 40 controls at T5.

[b]Fifty-four cases and 61 controls at T1–T3, 38 cases and 38 controls at T4, 20 cases and 28 controls at T5.

*$p < .05$.; **$p < .01$.; ***$p < .001$.

variables. A random intercept at the participant level was included to control for repeated measurements over time, with an unstructured variance and covariance correlation structure for within-group errors.

We assessed the associations between pre-diagnostic blood biomarker concentrations and T2DM. Logistic regression analyses were used to estimate odds ratios of T2DM for each time point separately. We fitted two models per blood biomarker: the first included blood biomarker concentration as a continuous, independent variable; in the second model, the blood biomarker was dichotomized according to clinical guidelines and concentrations associated with an increased risk of T2DM. Both models were adjusted for established risk factors, and odds ratios were estimated either per 1-unit increase in blood biomarker concentration or above versus below the defined clinical cut-off values: triglycerides >1.70 g/L, HDL <1.30 mmol/L for women and <1.03 for men,[30] total cholesterol >5.00 mmol/L, LDL >3.00 mmol/L[31] and HbA_{1c} >39.0 mmol/mol (5.7%).[18] Cut-offs for blood biomarkers with no clinical guidelines were based on a receiver operating characteristics curve (ROC) analysis in pre-diagnostic samples, which yielded the highest discrimination between cases and controls, and were as follows: total lipids >7.40 g/L for women (62.7% sensitivity, 63.3% specificity) and >7.59 for men (61.5% sensitivity, 61.5% specificity), free T_3 >5.20 pmol/L for women (33.0% sensitivity, 80.4% specificity) and >5.12 for

Post-diagnostic time points					
T3 (2001)		T4 (2007/08)		T5 (2015/16)	
Mean (SD)	ΔMean case-control (95% CI)	Mean (SD)	ΔMean case-control (95% CI)	Mean (SD)	ΔMean case-control (95% CI)
60.3 (6.31)	1.46 (−1.48, 4.39)	65.9 (6.39)	0.26 (−2.66, 3.18)	73.4 (6.07)	2.92 (−1.34, 7.19)
58.9 (8.98)		65.6 (7.83)		70.5 (9.71)	
63.4 (8.61)	2.05 (−1.57, 5.66)	68.5 (6.97)	1.61 (−2.37, 5.58)	72.6 (7.82)	2.34 (−3.43, 8.10)
61.4 (10.7)		66.9 (10.2)		70.2 (11.0)	
2.81 (1.54)	0.23 (−0.32, 0.79)	2.72 (1.45)	−0.09 (−0.70, 0.52)	2.62 (1.36)	−0.11 (−0.94, 0.73)
2.58 (1.45)		2.81 (1.52)		2.73 (1.83)	
15.1 (12.2)	−0.71 (−5.59, 4.18)	14.7 (13.5)	0.22 (−8.41, 3.73)	11.4 (8.57)	−8.70 (−15.3, −2.10)*
15.8 (11.5)		17.0 (12.4)		20.1 (13.5)	
81.9 (15.0)	12.2 (7.23, 17.2)***	81.7 (15.8)	12.6 (6.70, 18.5)***	81.6 (18.3)	11.8 (3.42, 20.2)**
69.7 (12.3)		69.1 (13.4)		69.8 (15.1)	
91.4 (14.0)	7.72 (2.98, 12.5)**	90.4 (12.0)	5.69 (0.41, 11.0)*	90.2 (14.5)	4.18 (−3.42, 11.8)
83.7 (11.6)		84.7 (10.9)		86.0 (11.7)	
31.8 (5.90)	5.22 (3.29, 7.15)***	31.8 (6.34)	5.05 (2.72, 7.37)***	31.5 (7.28)	4.64 (1.39, 7.89)**
26.5 (4.72)		26.7 (5.19)		26.9 (5.87)	
29.8 (3.58)	3.29 (1.99, 4.59)***	29.4 (3.45)	2.50 (0.95, 4.05)**	29.6 (3.79)	2.32 (0.19, 4.45)*
26.6 (3.46)		26.9 (3.29)		27.2 (3.49)	
96.1 (12.4)	12.0 (7.30, 16.8)***	103 (12.9)	12.7 (7.40, 17.9)***	105 (14.8)	14.7 (7.55, 21.8)***
84.1 (13.2)		90.1 (12.5)		90.0 (13.6)	
104 (9.43)	7.80 (4.23, 11.4)***	106 (8.66)	5.02 (0.81, 9.23)*	108 (12.5)	5.85 (−0.73, 12.4)
95.8 (9.87)		101 (9.36)		102 (10.1)	
85.1 (14.7)	6.97 (2.16, 11.8)**	79.2 (10.2)	2.37 (−1.86, 6.61)	71.0 (10.4)	−3.91 (−9.61, 1.80)
78.2 (11.7)		76.8 (10.5)		74.9 (11.9)	
83.2 (11.6)	−0.74 (−5.57, 4.09)	78.2 (12.0)	−4.22 (−9.33, 0.89)	72.8 (9.81)	−7.16 (−13.3, −1.05)*
84.0 (14.2)		82.4 (10.1)		80.0 (10.8)	
146 (21.0)	11.3 (3.34, 19.2)**	154 (25.4)	6.39 (−4.22, 16.9)	140 (25.4)	0.96 (−11.5, 13.4)
135 (22.1)	1.26 (−6.40, 8.93)	147 (26.5)		139 (24.4)	
143 (20.5)		144 (24.4)	2.19 (−8.44, 12.8)	132 (18.1)	−7.58 (−18.7, 3.52)
142 (20.6)		142 (21.6)		139 (19.4)	

men (54.6% sensitivity, 59.0% specificity), free T_4 <14.8 pmol/l for women (26.0% sensitivity, 53.6% specificity) and <14.0 for men (50.9% sensitivity, 34.7% specificity), TSH >1.92 mIU/L for women (47.0% sensitivity, 60.9 specificity) and >1.85 for men (61.1% sensitivity, 44.3% specificity), glucose >5.78 mmol/L for women (38.5% sensitivity, 91.3% specificity) and >5.59 for men (41.2% sensitivity, 77.3% specificity), and GGT >20.0 U/L for women (46.0% sensitivity, 83.9% specificity) and >25.0 for men (63.0% sensitivity, 68.9% specificity).

We assessed the following models: (1) a logistic regression model for established risk factors (age, BMI, physical activity, elevated blood pressure, family history of T2DM); (2) a blood biomarker model based on the significant blood biomarkers ($p < .05$) from the univariable unadjusted models, which were further reduced by a backwards selection process with best model fit as the selection criteria; and (3) a combined model including both established risk factors and blood biomarkers, using the same selection process as for the blood biomarker model. Model fit was assessed by Akaike's information criterion (AIC). Model discrimination was used to determine predictive value, assessed by area under the receiver operating characteristics (AROC). As per Hosmer and Lemeshow, an AROC of 0.50 indicates no discrimination, 0.50–0.70 poor discrimination, 0.70–0.80 acceptable discrimination, 0.80–0.90 excellent discrimination and ≥0.90 outstanding discrimination.[32]

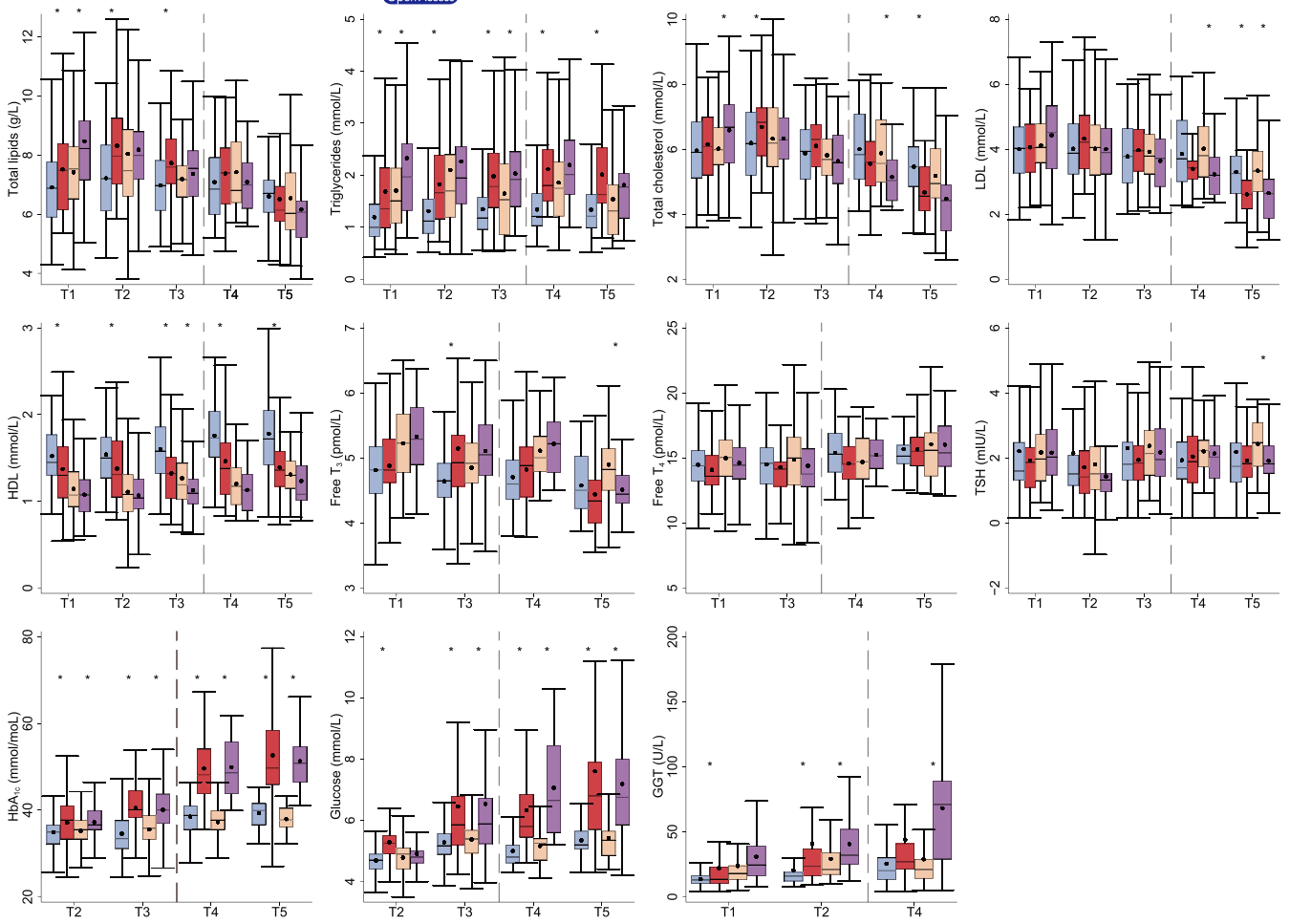


FIGURE 2 Pre- and post-diagnostic blood biomarker concentrations across surveys in female cases (red) and controls (blue) and male cases (purple) and controls (orange). Sample number for females were: 50 cases and 69 controls at T1–T3, 44 cases and 53 controls at T4, 26 cases and 40 controls at T5; and for males: 54 cases and 61 controls at T1–T3, 38 cases and 38 controls at T4, 20 cases and 28 controls at T5. HbA_{1c}, Glycated haemoglobin; HDL, High-density lipoprotein; GGT, Gamma-glutamyltransferase; LDL, Low-density lipoprotein; T, Time point; T_3, Triiodothyronine; T_4, Thyroxine; T2DM, type 2 diabetes mellitus; TSH, Thyroid-stimulating hormone. The Tromsø Study 1986–2016

Statistical analyses were performed in STATA (v. 17, StataCorp LLC, 4905 Lakeway Drive, College Station). All statistical analyses were stratified by sex, *p* values were two-sided, and a 5% level of significance was used.

3 | RESULTS

3.1 | Study sample characteristics

Type 2 diabetes mellitus cases and controls were similar in age, whereas cases were heavier, had higher BMI, and larger waist circumference (except men at T5) at all time points (Table 1). At pre-diagnostic time points, female cases had significantly higher blood pressure than controls, except for systolic blood pressure at T2. We observed no significant differences in blood pressure for males, except at T5, when cases had significantly lower diastolic blood pressure. In general, there were no differences in alcohol consumption or physical activity between cases and controls (Table S1), and no significant differences in parity or duration of breastfeeding between female cases and controls (Table 1). Female cases reported a family history of T2DM more frequently than female controls (Table S1).

Female cases had significantly higher triglyceride, HbA_{1c}, and glucose concentrations, and lower HDL concentrations than controls at all time points. Female cases also had significantly higher pre-diagnostic total lipids, total cholesterol (T2), free T_3 (T3) and GGT (T1–T2) concentrations than controls (Figure 2 and Table S2). However, post-diagnostic total cholesterol and LDL concentrations were significantly lower in cases than controls. Similarly, male cases had higher HbA_{1c} and glucose (except at T2) concentrations than controls at all time points. Further, male cases had higher pre-diagnostic total lipid (T1), triglyceride (T1 and T3), total cholesterol (T1), and GGT (T2) concentrations, and lower HDL concentrations (T3) than controls. Finally, post-diagnostic total cholesterol (T4), free T_3 (T5) and TSH (T5) concentrations were significantly higher in cases than controls.

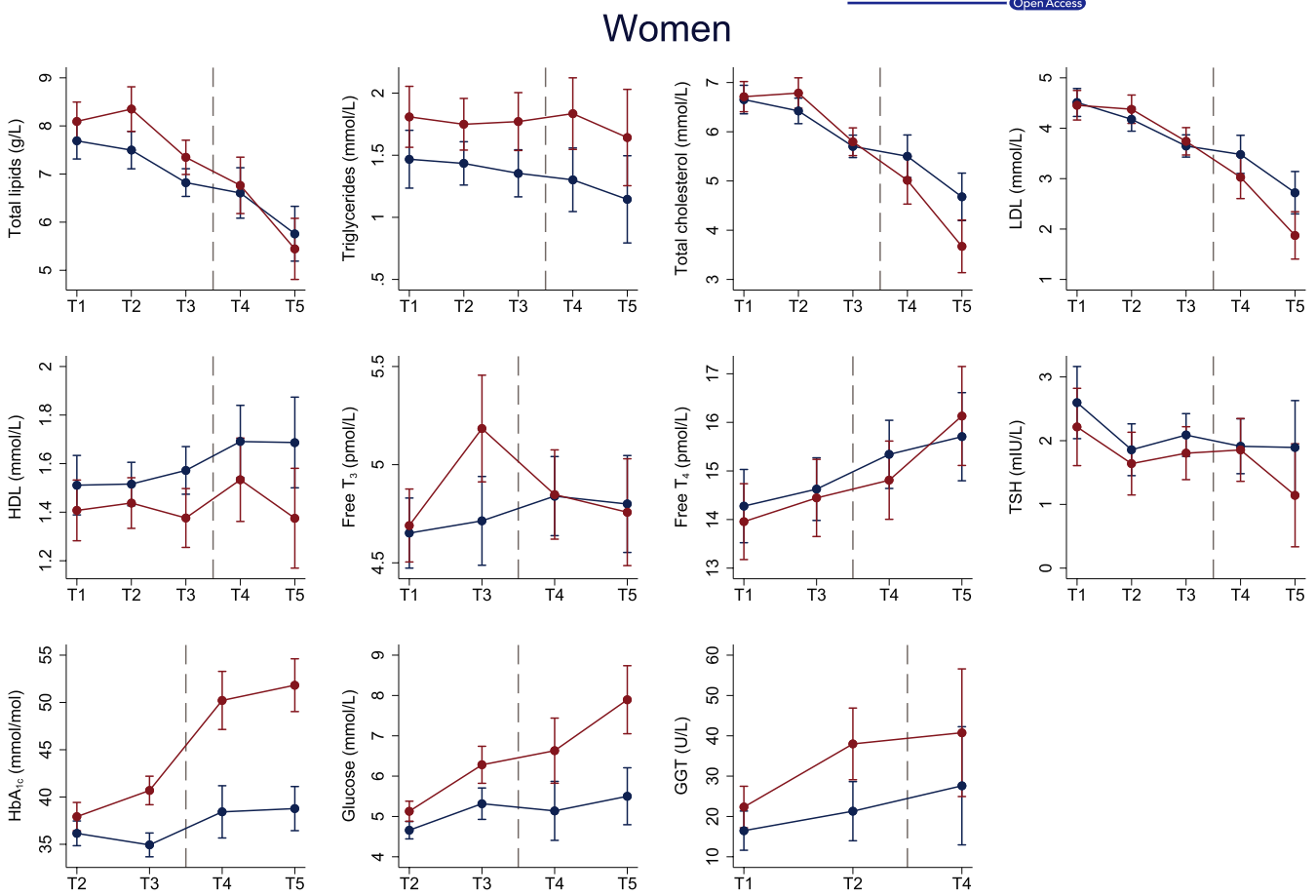


FIGURE 3 Estimated mean pre- and post-diagnostic blood biomarker concentrations (y-axis) across up to five time points (x-axis) for female cases (red) and controls (blue). Sample numbers: 50 cases and 69 controls at T1–T3, 44 cases and 53 controls at T4, 26 cases and 40 controls at T5. Models are adjusted for age, BMI, physical activity, elevated blood pressure and family history of type 2 diabetes. Dots represent mean concentrations and whiskers the 95% CI around the mean. HbA_{1c}, Glycated haemoglobin; HDL, High-density lipoprotein; GGT, Gamma-glutamyltransferase; LDL, Low-density lipoprotein; NA, not available; T, Time point; T_3, Triiodothyronine; T_4, Thyroxine; T2DM, type 2 diabetes mellitus; TSH, Thyroid-stimulating hormone. The Tromsø Study 1986–2016

3.2 | Longitudinal changes in blood biomarkers

After adjusting for age, BMI, physical activity, elevated blood pressure and family history of T2DM, female cases experienced a significantly larger increase in pre-diagnostic free T_3 (T1–T3), HbA_{1c} (T2-T3) and GGT (T1-T2) concentrations compared to controls (Figure 3 and Table S3). Further, there was a significantly larger increase in HbA_{1c} concentrations, and a larger decrease in total cholesterol, LDL and free T_3 concentrations in cases compared to controls from T3–T5.

Male cases experienced a significantly larger decrease in pre-diagnostic total lipid, total cholesterol, and LDL concentrations compared to controls, whereas significantly larger increases in HbA_{1c} and glucose concentrations were observed from T2–T3 in cases (Figure 4 and Table S3). Further, there was a significantly larger increase in post-diagnostic HbA_{1c} and HDL concentrations, and a larger decrease in free T_3 concentrations in cases compared to controls from T3–T5.

3.3 | Associations between pre-diagnostic blood biomarker concentrations and T2DM

In women, pre-diagnostic concentrations above the predefined cut-offs for HDL (T1) and free T_4 (T3) were inversely associated with T2DM, while total lipids and free T_3 (T3); triglycerides, HbA_{1c} and glucose (T2 and T3); and GGT (T2) were positively associated with T2DM after adjusting for established risk factors (Table S4). Further, HDL (T3), HbA_{1c} (T2 and T3), GGT (T2), total lipids (T3), triglycerides (T3) and free T_3 (T3) were associated with T2DM in a linear, dose-response manner. For men, concentrations above the predefined cut-offs for HbA_{1c} (T2 and T3), GGT (T2), total lipids, free T_3 and non-fasting glucose (T3) were positively associated with T2DM (Table S5). HbA_{1c} and glucose (T3) displayed a linear, dose-response relationship with T2DM.

At T1, the established risk factors model showed a higher predictive ability than the blood biomarker model for both men and women, while at T2 and T3, the blood biomarker model performed

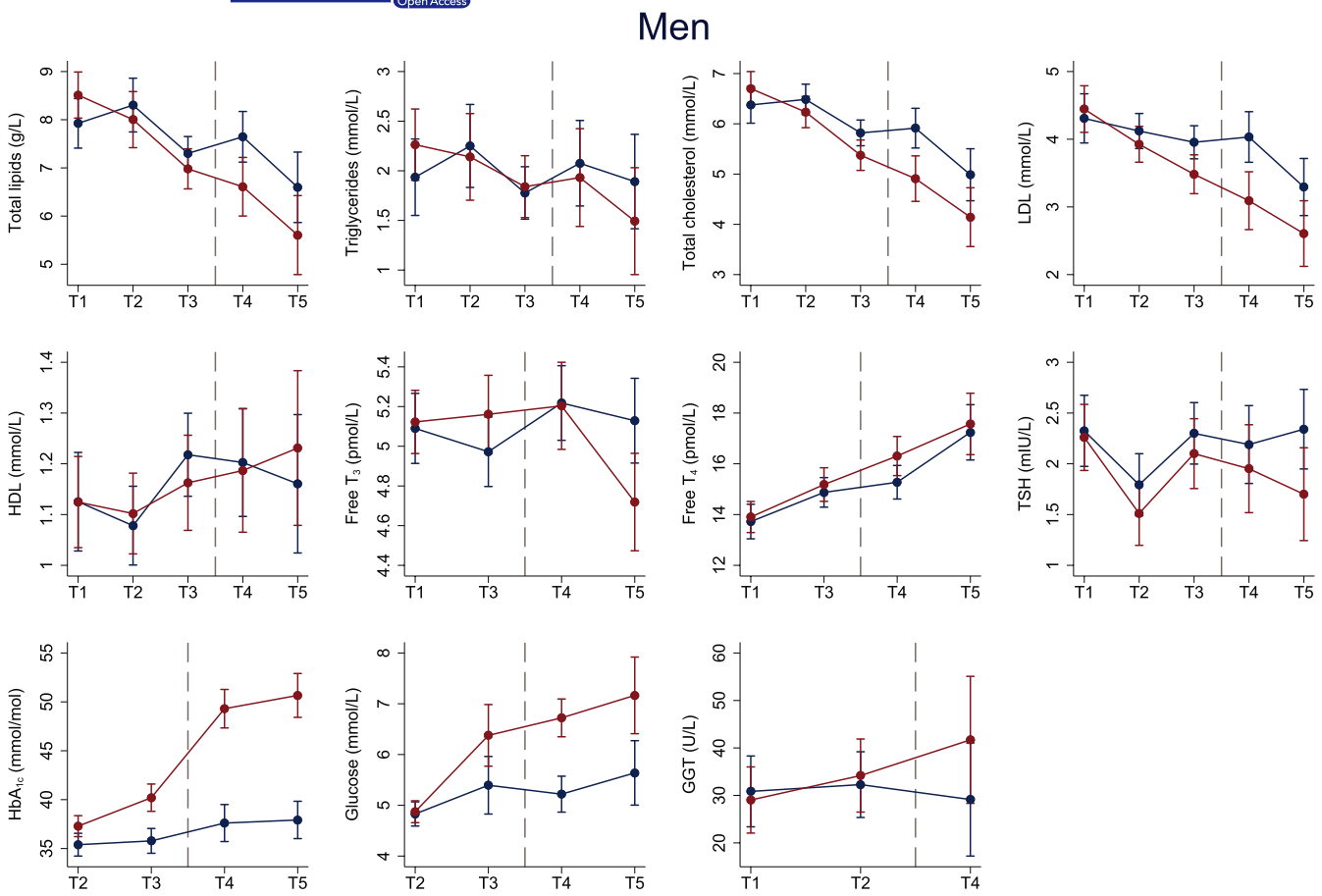


FIGURE 4 Estimated mean pre- and post-diagnostic blood biomarker concentrations (y-axis) across up to five time points (x-axis) for male cases (red) and controls (blue). Sample numbers: 54 cases and 61 controls at T1–T3, 38 cases and 38 controls at T4, 20 cases and 28 controls at T5. Models are adjusted for age, BMI, physical activity, elevated blood pressure, and family history of type 2 diabetes. Dots represent mean concentrations and whiskers the 95% CI around the mean. HbA_{1c}, Glycated haemoglobin; HDL, High-density lipoprotein; GGT, Gamma-glutamyltransferase; LDL, Low-density lipoprotein; NA, not available; T, Time point; T_3, Triiodothyronine; T_4, Thyroxine; T2DM, type 2 diabetes mellitus; TSH, Thyroid-stimulating hormone. The Tromsø Study 1986–2016

better (Tables 2 and 3). However, the combined model had increased predictive ability at every pre-diagnostic time point. The strongest discrimination between cases and controls was observed at T2 (95% for women and 85% for men), when the models for men and women were similar but not identical, as HDL was included for women only. Excluding HDL reduced discrimination among women to 94%, with a small loss of model fit (AIC 77.1 vs. 76.4).

4 | DISCUSSION

In this nested case–control study, we observed differences between cases and controls in total lipids, triglycerides, total cholesterol, HbA_{1c}, glucose and GGT that were present 15 years before T2DM diagnosis in cases. The model including established risk factors (age, BMI, physical activity, blood pressure and family history of T2DM) was sufficient to acceptably discriminate between cases and controls as early as 15 years before diagnosis (AROC: 0.73 for men and 0.76 for women), but discrimination increased in the combined model, which added blood biomarkers (0.78 and 0.79, respectively). The blood biomarker model displayed better predictive ability than the established risk factor model 7 years before diagnosis in cases (T2, AROC: 0.78 versus 0.73 in men and 0.88 versus 0.83 in women), but the combined model gave excellent predictive ability for men (AROC: 0.85) and outstanding predictive ability for women (AROC: 0.95). These findings suggest that several biomarkers of metabolic homeostasis, alone or combined with basic clinical information, can be used to predict T2DM up to 7 years before diagnosis. These blood biomarkers can be analysed easily and cost-effectively and provide objective measures. This approach could help identify high-risk individuals early, allowing preventive interventions to be implemented.

Our results showed that, regardless of the pre-diagnostic time point, a prediction model combining easily obtainable blood biomarkers and basic clinical information provided excellent predictive ability, even when different biomarkers are included. Using repeated measurements, we revealed that blood biomarkers have the potential to consistently predict disease 15 years before diagnosis. Our results are in agreement with other studies that used a single blood sample collected 5–10 years before T2DM diagnosis.[3,20-22,33,34] Although these studies included different basic

TABLE 2 Multivariable prediction of type 2 diabetes according to established risk factors and blood biomarkers across pre-diagnostic time points in women. The Tromsø Study 1986–2016

T1 (1986/87)			T2 (1994/95)			T3 (2001)		
	OR (95% CI)	AROC		OR (95% CI)	AROC		OR (95% CI)	AROC
Established risk factors[a]								
BMI ≥25 <30 (kg/m^2)	3.19 (1.23, 8.27)	0.76	BMI ≥25 < 30 (kg/m^2)	5.60 (1.70, 18.5)	0.83	BMI ≥25 < 30 (kg/m^2)	4.46 (1.23, 16.2)	0.80
BMI ≥30 (kg/m^2)	6.26 (1.86, 21.0)		BMI ≥30 (kg/m^2)	15.3 (3.98, 58.7)		BMI ≥30 (kg/m^2)	11.6 (3.05, 43.9)	
Physical activity (active)	1.20 (0.43, 3.34)		Physical activity (active)	0.36 (0.15, 0.90)		Physical activity (active)	0.40 (0.14, 1.12)	
Elevated blood pressure (yes)	2.19 (0.91, 5.27)		Elevated blood pressure (yes)	1.34 (0.48, 3.73)		Elevated blood pressure (yes)	2.91 (1.03, 8.19)	
Family history of T2DM (yes)	4.21 (1.28, 13.8)		Family history of T2DM (yes)	7.77 (2.40, 25.2)		Family history of T2DM (yes)	3.03 (1.12, 8.15)	
Blood biomarker[b]								
HDL ≥1.29 (mmol/L)	0.36 (0.16, 0.80)	0.66	Total lipids ≥7.40 (g/L)	3.88 (1.21, 12.5)	0.88	Triglycerides ≥1.70 (mmol/L)	5.69 (1.74, 18.7)	0.90
GGT ≥20.0 (U/L)	3.31 (1.12, 9.73)		HDL ≥1.29 (mmol/l)	0.47 (0.15, 1.47)		Free T_3 ≥5.20 (pmol/L)	3.15 (0.77, 12.9)	
			HbA_{1c} ≥39.0 (mmol/moL)	15.2 (3.28, 70.8)		Free T_4 ≥14.8 (pmol/L)	0.34 (0.10, 1.17)	
			GGT ≥20.0 (U/L)	18.3 (5.03, 66.4)		HbA_{1c} ≥39.0 (mmol/mol)	9.82 (3.03, 31.8)	
						Glucose ≥5.78 (mmol/L)	5.87 (1.68, 20.5)	
Combined model[ab]								
HDL ≥1.29 (mmol/L)	0.39 (0.16, 0.96)	0.79	HDL ≥1.29 (mmol/L)	0.29 (0.06, 1.35)	0.95	Triglycerides ≥1.70 (mmol/L)	7.28 (1.66, 31.9)	0.94
GGT ≥20.0 (U/L)	2.70 (0.81, 9.03)		HbA_{1c} ≥39.0 (mmol/mol)	39.7 (3.65, 432)		Free T_4 ≥14.8 (pmol/L)	0.23 (0.05, 1.02)	
BMI ≥25 <30 (kg/m^2)	3.06 (1.13, 8.25)		GGT ≥20.0 (U/l)	24.2 (3.65, 143)		HbA_{1c} ≥39.0 (mmol/mol)	13.3 (3.21, 55.0)	
BMI ≥30 (kg/m^2)	4.52 (1.24, 16.5)		BMI ≥25 <30 (kg/m^2)	11.4 (1.63, 79.8)		Glucose ≥5.78 (mmol/L)	3.25 (0.73, 14.4)	
Physical activity (active)	1.28 (0.43, 3.75)		BMI ≥30 (kg/m^2)	16.9 (2.00, 142)		BMI ≥25 <30 (kg/m^2)	1.00 (0.17, 5.94)	
Elevated blood pressure (yes)	2.12 (0.85, 5.27)		Physical activity (active)	0.19 (0.04, 0.85)		BMI ≥30 (kg/m^2)	6.72 (1.08, 41.9)	
Family history of T2DM (yes)	3.91 (1.16, 13.2)		Elevated blood pressure (yes)	0.92 (0.15, 5.59)		Physical activity (active)	0.30 (0.06, 1.45)	
			Family history of T2DM (yes)	28.6 (3.66, 224)		Elevated blood pressure (yes)	3.22 (0.68, 15.3)	
						Family history of T2DM (yes)	2.25 (0.54, 9.36)	

Abbreviations: AROC, Area under the receiver operating characteristic curve; BMI, Body mass index; GGT, Gamma-glutamyltransferase; HbA_{1c}, Glycated haemoglobin; HDL, High-density lipoprotein; T, Time point; T2DM, type 2 diabetes mellitus; T_3, Triiodothyronine; T_4, Thyroxine.

[a]Models were adjusted for age.

[b]Models were selected with backwards selection process according to best model fit.

TABLE 3 Multivariable prediction of type 2 diabetes according to established risk factors and blood biomarkers across pre-diagnostic time points in men. The Tromsø Study 1986–2016

T1 (1986/87)			T2 (1994/95)			T3 (2001)		
	OR (95%-CI)	AROC		OR (95%-CI)	AROC		OR (95%-CI)	AROC
Established risk factors[a]								
BMI ≥25 <30 (kg/m^2)	5.75 (2.31, 14.3)	0.73	BMI ≥25 < 30 (kg/m^2)	3.59 (1.31, 9.84)	0.73	BMI ≥25 < 30 (kg/m^2)	3.91 (1.16, 13.2)	0.71
BMI ≥0 (kg/m^2)	8.02 (2.03, 31.7)		BMI ≥30 (kg/m^2)	18.5 (4.34, 78.5)		BMI ≥30 (kg/m^2)	13.6 (3.25, 56.5)	
Physical activity (active)	0.71 (0.23, 2.18)		Physical activity (active)	0.92 (0.36, 2.35)		Physical activity (active)	1.15 (0.43, 3.06)	
Elevated blood pressure (yes)	1.29 (0.50, 3.29)		Elevated blood pressure (yes)	0.96 (0.35, 2.62)		Elevated blood pressure (yes)	0.57 (0.19, 1.66)	
Family history of T2DM (yes)	1.47 (0.51, 4.25)		Family history of T2DM (yes)	1.09 (0.41, 2.88)		Family history of T2DM (yes)	1.10 (0.44, 2.77)	
Blood biomarker[b]								
Total lipids ≥7.59 (g/L)	2.51 (1.07, 5.89)	0.72	HbA_{1c} ≥39.0 (mmol/mol)	14.2 (2.81, 71.3)	0.78	Triglycerides ≥1.70 (mmol/l)	2.77 (1.04, 7.34)	0.79
Triglycerides ≥1.70 (mmol/l)	1.76 (0.75, 4.12)		GGT ≥25 (U/L)	14.4 (3.88, 53.1)		HbA_{1c} ≥39.0 (mmol/mol)	5.39 (2.11, 13.8)	
GGT ≥25 (U/L)	2.91 (1.25, 6.75)					Glucose ≥5.59 (mmol/L)	3.24 (1.22, 8.59)	
Combined model[ab]								
Total lipids ≥7.59 (g/L)	2.04 (0.81, 5.12)	0.78	HbA_{1c} ≥39.0 (mmol/mol)	12.9 (2.22, 75.3)	0.85	Triglycerides ≥1.70 (mmol/l)	3.93 (1.15, 13.4)	0.84
GGT ≥25 (U/l)	2.26 (0.89, 5.74)		GGT ≥25 (U/l)	11.6 (2.61, 51.6)		HbA_{1c} ≥39.0 (mmol/mol)	8.50 (2.59, 27.9)	
BMI ≥25 <30 (kg/m^2)	3.89 (1.47, 10.3)		BMI ≥25 <30 (kg/m^2)	2.08 (0.55, 7.77)		Glucose ≥5.59 (mmol/L)	3.17 (1.02, 9.81)	
BMI ≥30 (kg/m^2)	3.96 (0.88, 17.7)		BMI ≥30 (kg/m^2)	20.0 (1.61, 248)		BMI ≥25 <30 (kg/m^2)	5.88 (1.25, 27.6)	
Physical activity (active)	0.72 (0.23, 2.28)		Physical activity (active)	2.23 (0.64, 7.81)		BMI ≥30 (kg/m^2)	14.9 (2.47, 89.7)	
Elevated blood pressure (yes)	1.28 (0.49, 3.37)		Elevated blood pressure (yes)	0.55 (0.14, 2.19)		Physical activity (active)	1.26 (0.34, 4.64)	
Family history of T2DM (yes)	1.64 (0.56, 4.83)		Family history of T2DM (yes)	1.25 (0.34, 4.65)		Elevated blood pressure (yes)	0.49 (0.14, 1.73)	
						Family history of T2DM (yes)	1.44 (0.40, 5.25)	

Abbreviations: AROC, Area under the receiver operating characteristic curve; BMI, Body mass index; GGT, Gamma-glutamyltransferase; HbA_{1c}, Glycated haemoglobin; T, Time point; T2DM, type 2 diabetes mellitus.

[a]Models were adjusted for age.

[b]Models were selected with backwards selection process according to best model fit.

clinical information, and sometimes different blood biomarkers, they all showed excellent discrimination (AROC: 0.78–0.90). They also displayed similar predictive abilities, although their biomarkers were different from ours, perhaps because their biomarkers were also related to prediabetic metabolic disturbances. For example, the prediction model proposed by the Framingham offspring study used personal information (age, sex, history of T2DM, BMI), blood pressure, HDL, triglycerides and fasting glucose and had excellent predictive ability (AROC: 0.85) 7 years before diagnosis.[20] Our prediction model for women at T2 (also 7 years before diagnosis) was very similar (e.g., personal information, blood pressure, total lipids, triglycerides and HDL), but we included GGT and HbA_{1c}, as fasting blood glucose was not available. As postprandial hyperglycaemia is more common in individuals with prediabetes,[35,36] fasting blood glucose may not identify disturbances in glucose homeostasis as well as HbA_{1c},[37] which may also explain the higher predictive ability of our models compared to the Framingham model. Further, our results are based on non-fasting blood samples, underlining the predictive value of non-fasting biomarkers, which would alleviate some of the restrictions of risk models based on fasting blood samples. Our results also complement studies that included repeated measurements collected from patient's healthcare records in models for predicting T2DM. The studies by Paprott et al.[38] and Pimentel et al.[39] concluded that risk factors such as lifestyle habits, BMI/waist circumference, hypertension and family history of diabetes, as well as temporal changes in these risk factors, successfully predicted future T2DM. The studies by Gurka et al.[40] and Bernardini et al.[41] observed that concentrations and temporal changes in concentrations of triglycerides, HDL, LDL, GGT and urea, strengthened their prediction models.

In the present study, all prediction models performed better in women than in men. Specifically, we observed stronger associations between lipids (total lipids, triglycerides and HDL), free T_3, free T_4, HbA_{1c}, glucose and T2DM in women than men. Several other studies (reviewed by Kautzky-Willer et al.[42]) demonstrated stronger associations between lipids and incident T2DM in women than men, possibly due to sex differences in fat deposition.[42] Njølstad et al.[43] also observed stronger associations between HDL, triglycerides, random glucose and T2DM in women than men in the Finnmark Study; BMI was a more important risk factor for men.

Many blood biomarkers were significant predictors of T2DM in our study; however, discrimination and model fit were not compromised even after several biomarkers were excluded from the models. This may be due to the very strong predictive abilities of some blood biomarkers. For example, at T2, HDL was significantly associated with T2DM among women after adjusting for established risk factors, but discrimination and model fit did not improve significantly in a model that included only HbA_{1c}, GGT and established risk factors. Unfortunately, we did not have GGT and HbA_{1c} at every pre-diagnostic time point and could not include them together at T1 and T3. However, we hypothesize that, had they been available, their combined inclusion would have improved the model discrimination at these time points as well. This is in line with previous findings that HbA_{1c} and GGT were on par with or better than a combination of other blood lipids and/or glucose measurements and significantly improved discrimination beyond established risk factors.[3,34] As such, for clinical purposes, our study showed that the inclusion of $HbA_{1c,}$ GGT and established risk factors would result in identical prediction models for men and women at all pre-diagnostic time points with excellent predictive ability.

Already at T2, HbA_{1c} concentrations were significantly higher in cases (~37 mmol/mol, 5.5%) than controls (~35 mmol/mol, 5.4%), though they were still within normal limits (42–47 mmol/mol, 6.0–6.4%) according to the International Expert Committee.[44] However, our results suggest that a lower HbA_{1c} threshold for risk assessment, one more in line with that recommended by the American Diabetes Association, may be warranted, as it would enable earlier identification of high-risk subjects. Our results are in line with studies on HbA_{1c} trajectories, which showed similar differences between cases and controls up to 10 years before diagnosis (cases: 37.0–40.0 mmol/mol, 5.5–5.8%; controls: 33.0–35.5 mmol/mol, 5.2–5.4%).[45,46]

We observed that cases had higher average GGT concentrations than controls and that men generally had higher concentrations than women. However, concentrations varied within the normal range of 10–75 U/l for women and 15–115 U/l for men.[31] This is in line with previous studies investigating liver biomarkers in relation to T2DM, which showed significantly higher GGT concentrations in cases than controls, and in men than women, though they remained within normal limits.[5,47,48] GGT has been identified as an independent risk factor for T2DM and is also linked to hepatic steatosis, which in turn is associated with obesity,[49] clearly emphasizing the potential of GGT as a predictive biomarker for T2DM.

Total cholesterol and LDL concentrations decreased in both cases and controls throughout the study period. A general decrease in cholesterol concentrations in the Tromsø Study from 1979 to 2016 was previously reported for both men and women.[50] The authors hypothesized that this was due to changes in cholesterol-associated lifestyle factors in the Norwegian population, such as a general increase in physical activity, and decreased smoking and consumption of trans fats. In our study, the steeper post-diagnostic decrease in cholesterol concentrations among cases may be explained by targeted lifestyle changes following the diagnosis, as individuals with T2DM have been shown to improve their lipid concentrations after diagnosis.[51] The decrease may also be attributed to the use of cholesterol-lowering drugs, as cardiovascular diseases are associated with T2DM. In our study, 43%–70% of cases and 5%–24% of controls reported using lipid-lowering drugs at T4 and T5, compared to 17%–40% in the general population within similar age groups and time periods.[50]

We observed different changes in free T_3 between cases and controls where cases generally had increased pre-diagnostic and decreased post-diagnostic concentrations. Free T_3 was positively associated with T2DM in men and women at T3, whereas free T_4 was inversely associated with T2DM in women at T3. This both agrees and disagrees with a recent meta-analysis including 12 prospective studies[52] that demonstrated positive associations between

TSH concentrations and T2DM, and inverse associations between free T_3 and free T_4 with T2DM. We did not observe any significant associations between TSH and T2DM, possibly due to small sample size. Time of blood sampling before diagnosis as well as study design might explain the different study observations. Accordingly, we observed that concentrations of free T_3 were similar between cases and controls at T1, with a notable increase in cases to T3, followed by a post-diagnostic decline. This observation is in line with the study by Jun et al.[53] where they observed an increased T_3 concentration at baseline followed by a decline over time in cases. This highlights that repeated measurements are important especially due to the properties of thyroid hormone homeostasis regulated by feedback mechanisms.[54,55] Discrete alterations in thyroid hormones may not be detected by measurement from a single time point and the interrelationship between levels of TSH, free T_3 and free T_4 and their associations with T2DM can be dependent on timing of measurements. There are very few longitudinal studies with repeated measurements of thyroid hormones with which we can compare our results to, and to our knowledge, none have presented repeated free T_3 measurements. Our observations may indicate an imbalance in thyroid homeostasis in T2DM cases, which may result in subclinical hyperthyroidism or hypothyroidism, and in turn, may affect insulin resistance and glucose concentrations.[56]

The main strength of this study is the nested case–control design with repeated measurements which allowed us to study pre- and post-diagnostic changes over 30 years, and produce prediction models for the same individuals at three different pre-diagnostic time points. Moreover, we had high-quality information for many clinical variables, possible confounding factors and a wide spectrum of relevant biomarkers. The design provided us with an important evolutionary overview of the biomarkers and how they relate to the progression of T2DM and beyond. Information on T2DM diagnosis was collected from local registries and laboratory data up until the last survey, and medical records were used to confirm that none of the controls had been diagnosed with T2DM.

After stratifying by sex, there were few observations at each time point among cases and controls, which limits the precision of our effect estimates. Due to a lack of serum, we were not able to analyse thyroid hormones at T2 nor glucose at T1; moreover, GGT was unavailable at T3 and T5, as was HbA_{1c} at T1. Waist circumference was also not available at T1, and only available for ~68% of subjects at T2. However, even though waist circumference has a stronger association with T2DM than BMI, it has not been shown to provide more accurate risk predictions of T2DM.[57] We had smaller sample sizes at post-diagnostic time points, as the inclusion criteria required an available blood sample at all pre-diagnostic ones. The prediction models were developed in a study sample from a northern Norwegian population, thus, the relative contribution of each predictor may vary in other populations due to genetical, environmental and lifestyle variations. Accordingly, our prediction models should be validated in different populations to verify their generalizability, and cut-offs should be re-evaluated if necessary.[19]

5 | CONCLUSIONS

Already 15 years before diagnosis, there were clear differences in blood biomarker concentrations between T2DM cases and controls and several blood biomarkers were associated with type T2DM. Selected blood biomarkers (lipids, HbA_{1c}, GGT) in combination with BMI, physical activity, elevated blood pressure, and family history of T2DM had excellent predictive ability 1–7 years before type 2 T2DM diagnosis and acceptable predictive ability up to 15 years before diagnosis.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Giovanni Allaoui: Data curation (lead); Formal analysis (lead); Methodology (lead); Writing – original draft (lead); Writing – review & editing (equal). **Charlotta Rylander:** Conceptualization (equal); Funding acquisition (supporting); Methodology (supporting); Project administration (equal); Supervision (supporting); Writing – original draft (supporting); Writing – review & editing (equal). **Maria Averina:** Supervision (supporting); Writing – original draft (supporting); Writing – review & editing (supporting). **Tom Wilsgaard:** Formal analysis (supporting); Methodology (supporting); Supervision (supporting); Writing – review & editing (supporting). **Ole-Martin Fuskevåg:** Methodology (supporting); Supervision (supporting); Writing – review & editing (supporting). **Vivian Berg:** Conceptualization (equal); Funding acquisition (lead); Methodology (supporting); Project administration (lead); Resources (lead); Supervision (lead); Writing – original draft (supporting); Writing – review & editing (equal).

DATA AVAILABILITY STATEMENT

The data set used in present study was derived from the Tromsø Study. It is not publicly available, but may be accessed through an application to the Tromsø Study (https://uit.no/research/tromsostudy).

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Supplemental material

Longitudinal changes in blood biomarkers and their ability to predict Type 2 Diabetes Mellitus – The Tromsø study

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Table S1. Characteristics of the study sample across five surveys of the Tromsø Study 1986-2016.

			Pre-diagnostic time-points			Post-diagnostic time-points	
			T1 1986/87	**T2 1994/95**	**T3 2001**	**T4 2007/08**	**T5 2015/16**
Alcohol consumption, n (%)							
Teetotaller	Women	Case	23 (50.0)	14 (28.0)	9 (18.4)	10 (24.4)	4 (15.4)
		Control	32 (47.8)	27 (39.1)	14 (20.6)	10 (19.2)	7 (18.0)
	Men	Case	15 (29.4)	10 (18.5)	8 (15.4)	6 (16.7)	3 (15.0)
		Control	12 (20.3)	4 (6.6)	5 (8.20)	4 (10.8)	1 (3.6)
1-4 times/ month	Women	Case	21 (45.7)	31 (62.0)	33 (67.4)	24 (58.5)	17 (65.4)
		Control	34 (50.8)	40 (58.0)	41 (60.3)	27 (51.9)	22 (56.4)
	Men	Case	31 (60.8)	40 (74.1)	34 (65.4)	26 (72.2)	14 (70.0)
		Control	39 (66.1)	43 (70.5)	41 (67.2)	25 (67.6)	16 (57.1)
> 4 times/ month	Women	Case	2 (4.4)	5 (10.0)	7 (14.3)	7 (17.1)	5 (19.2)
		Control	1 (1.5)	2 (2.9)	13 (19.1)	15 (28.9)	10 (25.6)
	Men	Case	5 (9.8)	4 (7.4)	10 (19.2)	4 (11.1)	3 (15.0)
		Control	8 (13.6)	14 (23.0)	15 (24.6)	8 (21.6)	11 (39.3)
Physical activity, n (%)							
Inactive	Women	Case	10 (20.0)	30 (60.0)	17 (34.7)	10 (25.6)	6 (26.1)
		Control	15 (21.7)	22 (31.9)	13 (19.4)	5 (10.9)	3 (8.8)
	Men	Case	9 (16.7)	16 (29.6)	11 (21.2)	9 (24.3)	10 (52.6)
		Control	10 (16.4)	15 (24.6)	16 (27.1)	8 (22.2)	2 (7.7)
Active	Women	Case	40 (80.0)	20 (40.0)	32 (65.3)	29 (74.4)	17 (73.9)
		Control	54 (78.3)	47 (68.1)	54 (80.6)	41 (89.1)	31 (91.2)
	Men	Case	45 (83.3)	38 (70.4)	41 (78.9)	28 (75.7)	9 (47.4)
		Control	51 (83.6)	46 (75.4)	43 (72.9)	28 (77.7)	24 (92.3)
Family history of type 2 diabetes, n (%)							
No	Women	Case	37 (74.0)	30 (60.0)	29 (58.0)	28 (63.6)	16 (61.5)
		Control	63 (91.3)	61 (88.4)	56 (81.2)	42 (79.3)	34 (85.0)
	Men	Case	43 (79.6)	41 (75.9)	38 (70.4)	26 (68.4)	13 (65.0)
		Control	51 (83.6)	48 (78.7)	46 (75.4)	29 (76.3)	23 (82.1)
Yes	Women	Case	13 (26.0)	20 (40.0)	21 (42.0)	16 (36.4)	10 (38.5)
		Control	6 (8.70)	8 (11.6)	13 (18.8)	11 (20.8)	6 (15.0)
	Men	Case	11 (20.4)	13 (24.1)	16 (29.6)	12 (31.6)	7 (35.0)
		Control	10 (16.4)	13 (21.3)	15 (24.6)	9 (23.7)	5 (17.9)
Elevated blood pressure, n (%)							
No	Women	Case	19 (38.0)	16 (32.0)	10 (20.0)	3 (6.82)	3 (11.5)
		Control	46 (66.7)	32 (46.4)	31 (44.9)	15 (28.3)	10 (25.0)
	Men	Case	12 (22.2)	10 (18.5)	11 (20.4)	5 (13.2)	3 (15.0)
		Control	20 (32.8)	16 (26.2)	14 (23.0)	10 (26.3)	7 (25.0)
Yes	Women	Case	31 (62.0)	34 (68.0)	40 (80.0)	41 (93.2)	23 (88.5)
		Control	23 (33.3)	37 (53.6)	38 (55.1)	38 (71.7)	30 (75.0)
	Men	Case	42 (77.8)	44 (81.5)	43 (79.6)	33 (86.8)	17 (85.0)
		Control	41 (67.2)	45 (73.8)	47 (77.1)	28 (73.7)	21 (75.0)
Us of lipid-lowering drugs, n (%)							
No	Women	Case	NA	49 (98.0)	41 (85.4)	25 (56.8)	10 (40.0)
		Control		68 (98.6)	60 (88.3)	43 (82.7)	26 (76.5)
	Men	Case		51 (94.4)	38 (73.1)	20 (52.6)	6 (30.0)
		Control		61 (100)	54 (91.5)	36 (94.7)	20 (76.9)
Yes	Women	Case	NA	1 (1.45)	7 (14.6)	19 (43.2)	15 (60.0)
		Control		1 (2.00)	8 (11.8)	9 (17.3)	8 (23.5)
	Men	Case		3 (5.56)	14 (26.9)	18 (47.4)	14 (70.0)
		Control		0 (0.00)	5 (8.47)	2 (5.26)	6 (23.1)

T: time-point.

Table S2. Pre- and post- diagnostic blood biomarker concentrations across five surveys in The Tromsø Study 1986-2016.

			Pre-diagnostic time-points						**Post-diagnostic time-points**			
			T1 1986/87		**T2 1994/95**		**T3 2001**		**T4 2007/08**		**T5 2015/16**	
Biomarker			**Mean (SD)**	**Median (5, 95 percentiles)**	**Mean (SD)**	**Median (5, 95 percentiles)**	**Mean (SD)**	**Median (5, 95 percentiles)**	**Mean (SD)**	**Median (5, 95 percentiles)**	**Mean (SD)**	**Median (5, 95 percentiles)**
Total lipids (g/L)	Women[a]	Case	7.52 (1.49)	7.48 (5.43, 9.98)	8.31 (2.10)	7.97 (5.87, 10.7)	7.73 (1.28)	7.59 (5.82, 9.79)	7.38 (1.50)	7.40 (5.01, 9.87)	6.51 (1.32)	6.13 (5.30, 9.22)
		Control	6.91 (1.42)	6.88 (4.81, 9.40)	7.22 (1.42)	7.21 (4.88, 9.77)	6.98 (1.20)	6.98 (5.04, 9.01)	7.09 (1.27)	6.86 (5.40, 9.16)	6.61 (0.86)	6.71 (5.46, 7.75)
	Men[b]	Case	8.47 (2.03)	8.23 (5.86, 12.2)	8.20 (1.64)	8.00 (5.95, 11.4)	7.37 (1.30)	7.56 (5.03, 9.53)	7.09 (1.04)	6.99 (5.67, 8.83)	6.16 (1.78)	6.06 (4.03, 10.7)
		Control	7.42 (1.34)	7.45 (5.22, 9.65)	8.04 (2.61)	7.48 (5.56, 10.8)	7.19 (1.43)	7.16 (5.03, 8.90)	7.43 (1.45)	6.81 (5.80, 10.3)	6.54 (1.60)	6.04 (4.75, 9.31)
Triglycerides (mmol/L)	Women	Case	1.69 (1.02)	1.36 (0.69, 4.28)	1.83 (2.10)	1.67 (0.76, 3.62)	1.98 (0.94)	1.78 (0.77, 3.66)	2.12 (0.85)	1.81 (1.30, 4.19)	2.01 (1.02)	1.63 (0.97, 3.50)
		Control	1.19 (0.55)	1.00 (0.59, 2.30)	1.31 (0.62)	1.12 (0.68, 2.67)	1.35 (0.58)	1.18 (0.72, 2.74)	1.34 (0.52)	1.21 (0.65, 2.31)	1.34 (0.51)	1.21 (0.70, 2.34)
	Men	Case	2.33 (1.64)	1.97 (0.94, 5.44)	2.27 (1.24)	1.95 (1.05, 4.99)	2.03 (0.94)	1.92 (0.97, 4.07)	2.20 (0.88)	2.02 (1.14, 4.01)	1.81 (0.88)	1.78 (0.82, 3.96)
		Control	1.71 (0.84)	1.51 (0.73, 3.22)	2.10 (1.93)	1.70 (0.81, 4.12)	1.65 (1.10)	1.54 (0.74, 2.82)	1.86 (1.08)	1.75 (0.61, 4.05)	1.54 (0.92)	1.32 (0.68, 3.84)
Total cholesterol (mmol/L)	Women	Case	6.16 (1.02)	6.09 (4.70, 7.94)	6.69 (1.21)	6.84 (5.03, 8.84)	6.11 (0.95)	6.31 (4.39, 7.48)	5.56 (1.21)	5.61 (3.67, 7.08)	4.68 (0.81)	4.56 (3.48, 5.94)
		Control	5.96 (1.26)	5.91 (3.76, 7.92)	6.20 (1.22)	6.18 (4.09, 8.09)	5.89 (1.05)	5.94 (4.20, 7.49)	6.01 (1.07)	5.84 (4.61, 7.87)	5.47 (0.95)	5.47 (4.07, 6.71)
	Men	Case	6.59 (1.28)	6.69 (4.60, 9.00)	6.33 (1.04)	6.29 (4.54, 8.32)	5.63 (0.95)	5.60 (3.99, 7.00)	5.15 (0.80)	5.03 (4.18, 6.57)	4.48 (1.30)	4.43 (2.76, 7.45)
		Control	6.02 (1.07)	5.98 (4.25, 8.02)	6.33 (1.32)	6.20 (4.36, 8.12)	5.82 (1.07)	5.67 (4.24, 7.95)	5.88 (1.24)	5.56 (4.27, 8.04)	5.19 (1.13)	4.95 (3.85, 6.84)
LDL (mmol/L)	Women	Case	4.06 (0.96)	4.03 (2.40, 5.58)	4.32 (1.09)	4.22 (2.80, 6.36)	3.97 (0.90)	4.00 (2.59, 5.41)	3.40 (0.93)	3.43 (1.67, 5.01)	2.61 (0.69)	2.61 (1.56, 3.70)
		Control	4.01(1.10)	4.03 (2.17, 5.69)	4.01 (1.06)	3.88 (2.18, 5.64)	3.78 (0.99)	3.78 (2.22, 5.54)	3.86 (1.07)	3.71 (2.39, 5.88)	3.30 (0.89)	3.26 (2.09, 4.78)
	Men	Case	4.43 (1.34)	4.50 (2.38, 6.91)	4.00 (1.04)	3.90 (2.43, 6.05)	3.64 (0.92)	3.72 (2.11, 4.96)	3.23 (0.73)	3.20 (2.41, 4.68)	2.65 (1.04)	2.64 (1.42, 4.94)
		Control	4.12 (1.01)	4.06 (2.53, 5.76)	4.02 (0.97)	4.06 (2.44, 5.53)	3.92 (1.00)	3.79 (2.56, 5.64)	4.02 (1.21)	3.81 (2.54, 6.24)	3.34 (0.93)	3.33 (1.95, 4.78)
HDL (mmol/L)	Women	Case	1.37 (0.41)	1.30 (0.86, 2.07)	1.39 (0.39)	1.31 (0.9, 2.24)	1.32 (0.37)	1.32 (0.84, 2.04)	1.46 (0.52)	1.38 (0.90, 2.24)	1.39 (0.41)	1.36 (0.76, 2.14)
		Control	1.52 (0.33)	1.45 (1.09, 2.13)	1.54 (0.33)	1.50 (1.03, 2.15)	1.60 (0.41)	1.58 (0.92, 2.32)	1.76 (0.42)	1.73 (1.12, 2.60)	1.78 (0.50)	1.72 (0.93, 2.58)
	Men	Case	1.08 (0.26)	1.08 (0.69, 1.56)	1.07 (0.23)	1.04 (0.67, 1.38)	1.13 (0.27)	1.10 (0.7, 1.71)	1.13 (0.29)	1.13 (0.79, 1.65)	1.23 (0.37)	1.08 (0.82, 2.12)
		Control	1.15 (0.36)	1.07 (0.72, 1.65)	1.11 (0.37)	1.08 (0.57, 1.71)	1.27 (0.40)	1.19 (0.76, 1.95)	1.20 (0.29)	1.18 (0.80, 1.74)	1.31 (0.25)	1.30 (0.85, 1.70)
Free T_3 (pmol/L)	Women	Case	4.88 (0.55)	4.82 (3.84, 5.60)	NA	NA	5.15 (1.30)	4.93 (4.06, 6.73)	4.82 (0.57)	4.89 (4.01, 5.60)	4.44 (0.63)	4.34 (3.71, 5.78)
		Control	4.81 (0.54)	4.81 (4.06, 5.53)			4.64 (0.54)	4.65 (3.78, 5.50)	4.71 (0.53)	4.60 (3.83, 5.78)	4.58 (0.45)	4.50 (3.90, 5.27)
	Men	Case	5.33 (0.56)	5.29 (4.38, 6.29)			5.10 (0.53)	5.05 (4.30, 6.12)	5.22 (0.53)	5.22 (4.52, 6.20)	4.51 (0.35)	4.45 (3.98, 5.16)
		Control	5.23 (0.60)	5.24 (4.37, 6.32)			4.85 (0.85)	4.93 (4.03, 6.03)	5.11 (0.50)	5.00 (4.46, 6.00)	4.90 (0.63)	4.83 (4.15, 6.15)
Free T_4 (pmol/L)	Women	Case	14.1 (2.00)	13.6 (11.2, 18.0)	NA	NA	14.3 (3.30)	14.2 (11.1, 17.2)	14.6 (1.87)	14.5 (11.8, 18.0)	15.7 (2.01)	15.5 (13.1, 18.2)
		Control	14.5 (2.39)	14.4 (10.9, 19.0)			14.5 (2.30)	14.6 (11.6, 17.9)	15.4 (2.08)	15.3 (11.8, 18.8)	15.7 (2.27)	15.2 (13.5, 20.8)
	Men	Case	14.6 (1.95)	14.5 (11.5, 18.4)			14.4 (2.37)	13.8 (10.8, 19.2)	15.3 (1.43)	15.2 (12.9, 17.5)	16.0 (2.22)	15.4 (12.7, 20.1)
		Control	15.0 (2.31)	15.0 (11.1, 18.1)			14.9 (2.44)	14.8 (11.5, 19.3)	14.7 (2.02)	14.7 (11.7, 17.0)	16.1 (2.97)	15.6 (12.3, 22.8)
TSH (mIU/L)	Women	Case	1.92 (0.95)	1.87 (0.82, 3.83)	1.71 (0.88)	1.41 (0.71, 3.41)	1.94 (0.97)	1.85 (0.75, 3.61)	2.04 (0.97)	1.89 (0.89, 3.68)	1.92 (0.74)	1.83 (0.98, 3.47)
		Control	2.22 (2.34)	1.60 (0.66, 4.12)	2.15 (2.50)	1.51 (0.89, 3.20)	2.30 (2.73)	1.81 (0.75, 4.96)	1.93 (0.86)	1.70 (0.77, 3.71)	2.19 (1.95)	1.74 (0.52, 6.48)
	Men	Case	2.17 (1.01)	2.03 (0.64, 4.08)	1.43 (0.71)	1.33 (0.66, 3.14)	2.17 (1.00)	1.96 (0.74, 3.81)	2.14 (1.19)	2.03 (0.55, 4.80)	1.91 (0.86)	1.82 (0.31, 3.55)
		Control	2.18 (1.14)	1.89 (0.70, 4.11)	1.81 (1.14)	1.50 (0.48, 3.45)	2.37 (1.34)	2.10 (0.81, 5.06)	2.20 (0.82)	2.23 (0.92, 3.61)	2.43 (0.83)	2.48 (1.16, 3.74)
Hba1c (mmol/mol)	Women	Case	NA	NA	37.1 (4.99)	37.7 (30.0, 43.2)	40.5 (4.76)	40.1 (32.1, 47.2)	50.7 (12.8)	47.0 (39.9, 65.0)	52.4 (8.72)	49.7 (42.1, 69.4)
		Control			34.9 (3.41)	35.0 (30.0, 41.0)	34.5 (4.71)	33.4 (27.2, 43.4)	38.9 (3.81)	39.9 (33.3, 45.3)	39.5 (3.03)	39.9 (34.4, 45.3)
	Men	Case			37.2 (3.57)	36.6 (33.3, 42.1)	40.1 (4.66)	40.0 (32.2, 46.9)	48.8 (7.52)	48.6 (37.7, 61.7)	51.3 (6.65)	50.8 (41.5, 65.6)
		Control			35.2 (3.22)	35.5 (30.0, 39.9)	35.6 (4.39)	35.8 (27.3, 43.2)	37.4 (3.42)	37.7 (31.1, 42.1)	38.0 (2.94)	37.7 (32.2, 42.1)
Glucose (mmol/L)	Women	Case	NA	NA	5.28 (0.73)	5.25 (4.40, 6.50)	6.46 (2.35)	5.86 (4.39, 10.3)	6.53 (3.42)	5.70 (4.70, 10.9)	7.54 (2.98)	6.70 (4.60, 13.5)
		Control			4.68 (0.57)	4.70 (3.80, 5.60)	5.28 (0.78)	5.17 (4.25, 6.58)	5.02 (0.58)	4.85 (4.40, 6.10)	5.31 (0.56)	5.20 (4.30, 6.80)
	Men	Case			4.91 (0.57)	4.80 (4.20, 5.90)	6.53 (3.17)	5.89 (4.47, 9.74)	6.85 (1.52)	6.60 (5.1, 10.2)	7.19 (2.19)	6.75 (4.30, 12.3)
		Control			4.78 (0.71)	4.90 (3.70, 6.10)	5.37 (0.76)	5.39 (4.17, 6.48)	5.17 (0.49)	5.20 (4.30, 6.00)	5.59 (0.99)	5.40 (4.70, 7.20)
GGT (U/L)	Women	Case	22.1 (23.4)	13.5 (7.00, 90.0)	40.8 (41.9)	23.5 (12.0, 109)	NA	NA	44.0 (47.7)	26.0 (13.0, 138)	NA	NA
		Control	13.6 (6.03)	13.0 (7.00, 28.0)	20.3 (18.3)	16.0 (9.00, 47.0)			26.4 (21.4)	19.5 (8.00, 84.0)		
	Men	Case	30.7 (26.1)	24.5 (10.0, 70.0)	40.5 (28.2)	32.0 (14.0, 84.0)			53.0 (39.8)	35.0 (13.0, 139)		
		Control	23.7 (24.0)	17.5 (8.00, 49.0)	29.2 (28.7)	21.0 (12.0, 57.0)			27.2 (20.3)	21.0 (12.5, 82.5)		

[a]50 cases and 69 controls at T1-T3, 44 cases and 53 controls at T4, 26 cases and 40 controls at T5.

[b]54 cases and 61 controls at T1-T3, 38 cases and 38 controls at T4, 20 cases and 28 controls at T5.

T: time-point; TSH: thyroid stimulating hormone; GGT: gamma-glutamyltransferase.

Table S3. Multivariable adjusted regression coefficients, standard error, and 95% CI from linear mixed effect models to assess longitudinal changes in biomarkers from 1986 to 2016 according to type 2 diabetes mellitus status. Tromsø Study 1986-2016.

Biomarker		Males[a]			Females[b]		
		β-coefficient (SE)	p-value	95% confidence interval	β-coefficient (SE)	p-value	95% confidence interval
Total lipids (g/L)	Case	-0.32 (0.27)	0.24	-0.85, 0.21	0.52 (0.23)	0.03	0.07, 0.98
	T1	0.62 (0.29)	0.03	0.06, 1.19	0.87 (0.22)	<0.01	0.43, 1.31
	T2	1.00 (0.26)	<0.01	0.49, 1.51	0.68 (0.21)	<0.01	0.27, 1.08
	T3	Reference	--	--	Reference	--	--
	T4	0.35 (0.22)	0.12	-0.09, 0.78	-0.22 (0.28)	0.43	-0.76, 0.32
	T5	-0.70 (0.32)	0.03	-1.33, -0.08	-1.06 (0.29)	<0.01	-1.64, -0.49
	Case#T1	0.91 (0.32)	<0.01	0.29, 1.53	-0.12 (0.26)	0.65	-0.63, 0.39
	Case#T2	0.02 (0.36)	0.96	-0.68, 0.72	0.33 (0.30)	0.27	-0.26, 0.91
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	-0.72 (0.32)	0.02	-1.34, -0.10	-0.37 (0.41)	0.37	-1.16, 0.43
	Case#T5	-0.67 (0.41)	0.10	-1.48, 0.14	-0.84 (0.41)	0.04	-1.64, -0.04
	Constant	2.76 (1.14)	0.02	0.52, 4.99	3.03 (0.70)	<0.01	1.65, 4.41
Triglycerides (mmol/L)	Case	0.06 (0.20)	0.76	-0.34, 0.46	0.42 (0.15)	0.01	0.12, 0.72
	T1	0.16 (0.22)	0.47	-0.27, 0.59	0.11 (0.14)	0.41	-0.16, 0.39
	T2	0.47 (0.20)	0.02	0.08, 0.86	0.08 (0.10)	0.41	-0.11, 0.27
	T3	Reference	--	--	Reference	--	--
	T4	0.30 (0.19)	0.12	-0.07, 0.67	-0.05 (0.13)	0.69	-0.31, 0.21
	T5	0.12 (0.20)	0.56	-0.27, 0.50	-0.21 (0.17)	0.21	-0.54, 0.12
	Case#T1	0.27 (0.24)	0.27	-0.21, 0.74	-0.08 (0.16)	0.63	-0.38, 0.23
	Case#T2	-0.17 0.28)	0.53	-0.71, 0.37	-0.10 (0.13)	0.44	-0.36, 0.16
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	-0.21 (0.27)	0.44	-0.74, 0.32	0.12 (0.19)	0.54	-0.26, 0.49
	Case#T5	-0.46 (0.23)	0.05	-0.91, -0.01	0.08 (0.22)	0.71	-0.35, 0.52
	Constant	-0.62 (0.84)	0.46	-2.27, 1.02	-0.24 (0.44)	0.59	-1.11, 0.63
Total Cholesterol (mmol/L)	Case	-0.44 (0.20)	0.02	-0.83, -0.06	0.09 (0.19)	0.61	-0.27, 0.46
	T1	0.56 (0.20)	0.01	0.17, 0.95	0.95 (0.18)	<0.01	0.60, 1.30
	T2	0.67 (0.15)	<0.01	0.38, 0.95	0.72 (0.15)	<0.01	0.43, 1.01
	T3	Reference	--	--	Reference	--	--
	T4	0.09 (0.17)	0.57	-0.23, 0.42	-0.20 (0.23)	0.37	-0.64, 0.24
	T5	-0.83 (0.25)	<0.01	-1.32, -0.35	-1.03 (0.25)	<0.01	-1.52, -0.53
	Case#T1	0.77 (0.21)	<0.01	0.35, 1.18	-0.03 (0.21)	0.87	-0.45, 0.38
	Case#T2	0.19 (0.19)	0.32	-0.18, 0.57	0.27 (0.21)	0.20	-0.14, 0.68
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	-0.56 (0.24)	0.02	-1.02, -0.1	-0.58 (0.33)	0.08	-1.23, 0.07
	Case#T5	-0.40 (0.33)	0.23	-1.07, 0.26	-1.10 (0.36)	<0.01	-1.79, -0.40
	Constant	3.02 (0.81)	<0.01	1.42, 4.61	2.49 (0.55)	<0.01	1.41, 3.56
LDL (mmol/L)	Case	-0.47 (0.19)	0.01	-0.84, -0.11	0.09 (0.18)	0.60	-0.26, 0.44
	T1	0.35 (0.19)	0.07	-0.02, 0.73	0.86 (0.17)	<0.01	0.53, 1.19
	T2	0.17 (0.12)	0.18	-0.07, 0.41	0.53 (0.14)	<0.01	0.26, 0.80
	T3	Reference	--	--	Reference	--	--
	T4	0.08 (0.16)	0.62	-0.23, 0.38	-0.17 (0.20)	0.40	-0.56, 0.23
	T5	-0.66 (0.20)	<0.01	-1.06, -0.26	-0.93 (0.22)	<0.01	-1.37, -0.49
	Case#T1	0.61 (0.21)	<0.01	0.21, 1.01	-0.15 (0.20)	0.45	-0.53, 0.24
	Case#T2	0.28 (0.16)	0.08	-0.03, 0.58	0.11 (0.19)	0.58	-0.28, 0.49
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	-0.47 (0.22)	0.03	-0.90, -0.04	-0.55 (0.29)	0.06	-1.12, 0.03
	Case#T5	-0.22 (0.27)	0.43	-0.75, 0.32	-0.94 (0.31)	<0.01	-1.56, -0.32
	Constant	2.15 (0.75)	<0.01	0.67, 3.63	1.35 (0.52)	0.01	0.33, 2.37
HDL (mmol/L)	Case	-0.06 (0.06)	0.37	-0.18, 0.05	-0.20 (0.08)	0.01	-0.35, -0.04
	T1	-0.09 (0.05)	0.06	-0.19, 0.02	-0.06 (0.07)	0.38	-0.20, 0.07
	T2	-0.14 (0.03)	<0.01	-0.20, -0.06	-0.06 (0.04)	0.18	-0.14, 0.03
	T3	Reference	--	--	Reference	--	--
	T4	-0.02 (0.04)	0.70	-0.09, 0.05	0.12 (0.06)	0.04	0.003, 0.23
	T5	-0.06 (0.05)	0.29	-0.16, 0.07	0.11 (0.08)	0.14	-0.04, 0.27
	Case#T1	0.05 (0.04)	0.19	-0.03, 0.14	0.09 (0.06)	0.12	-0.02, 0.21
	Case#T2	0.08 (0.04)	0.05	0.001, 0.16	0.12 (0.05)	0.02	0.02, 0.21
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	0.04 (0.05)	0.47	-0.07, 0.19	0.04 (0.08)	0.64	-0.12, 0.20
	Case#T5	0.13 (0.06)	0.05	0.001, 0.25	-0.12 (0.09)	0.19	-0.29, 0.06
	Constant	1.61 (0.22)	<0.01	1.17, 2.04	1.83 (0.25)	<0.01	1.34, 2.32

Free T3 (pmol/L)	Case	0.19 (0.13)	0.16	-0.07, 0.45	0.47 (0.18)	0.01	0.11, 0.83
	T1	0.12 (0.11)	0.30	-0.11, 0.34	-0.06 (0.14)	0.66	-0.34, 0.22
	T3	Reference	--	--	Reference	--	--
	T4	0.25 (0.12)	0.04	0.01, 0.48	0.13 (0.14)	0.37	-0.15, 0.40
	T5	0.16 (0.10)	0.12	-0.04, 0.36	0.09 (0.14)	0.53	-0.18, 0.35
	Case#T1	-0.16 (0.13)	0.24	-0.42, 0.11	-0.43 (0.19)	0.02	-0.80, -0.06
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	-0.20 (0.17)	0.24	-0.54, 0.14	-0.46 (0.21)	0.03	-0.87, -0.05
	Case#T5	-0.60 (0.13)	<0.01	-0.85, -0.35	-0.51 (0.18)	0.01	-0.87, -0.16
	Constant	5.24 (0.42)	<0.01	4.43, 6.06	5.49 (0.36)	<0.01	4.78, 6.20
Free T4 (pmol/L)	Case	0.31 (0.44)	0.49	-0.56, 1.18	-0.18 (0.53)	0.73	-1.21, 0.85
	T1	-1.15 (0.35)	<0.01	-1.83, -0.47	-0.35 (0.44)	0.43	-1.22, 0.52
	T3	Reference	--	--	Reference	--	--
	T4	0.40 (0.28)	0.15	-0.14, 0.94	0.72 (0.39)	0.07	-0.05, 1.48
	T5	2.37 (0.47)	<0.01	1.44, 3.29	1.08 (0.46)	0.02	0.18, 1.96
	Case#T1	-0.13 (0.29)	0.66	-0.70, 0.44	-0.14 (0.49)	0.78	-1.10, 0.82
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	0.73 (0.39)	0.06	-0.04, 1.49	-0.35 (0.56)	0.53	-1.44, 0.74
	Case#T5	0.02 (0.59)	0.97	-1.14, 1.19	0.61 (0.57)	0.29	-0.52, 1.73
	Constant	23.2 (1.61)	<0.01	20.1, 26.4	15.8 (1.46)	<0.01	13.0, 18.7
TSH (mIU/L)	Case	-0.20 (0.23)	0.39	-0.65, 0.25	-0.29 (0.27)	0.29	-0.82, 0.25
	T1	0.02 (0.18)	0.90	-0.33, 0.38	0.51 (0.26)	0.05	0.002, 1.02
	T2	-0.51 (0.16)	<0.01	-0.83, -0.18	-0.23 (0.18)	0.20	-0.58, 0.12
	T3	Reference	--	--	Reference	--	--
	T4	-0.11 (0.17)	0.51	-0.44, 0.22	-0.18 (0.18)	0.31	-0.52, 0.17
	T5	0.04 (0.20)	0.84	-0.35, 0.43	-0.20 (0.32)	0.55	-0.83, 0.44
	Case#T1	0.13 (0.18)	0.46	-0.22, 0.49	-0.10 (0.23)	0.66	-0.54, 0.34
	Case#T2	-0.08 (0.22)	0.71	-0.52, 0.35	0.07 (0.24)	0.78	-0.41, 0.55
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	0.04 (0.23)	0.87	-0.50, 0.42	0.23 (0.23)	0.33	-0.23, 0.68
	Case#T5	-0.44 (0.27)	0.10	-0.97, 0.09	-0.47 (0.38)	0.22	-1.21, 0.27
	Constant	1.40 (0.77)	0.07	-0.12, 2.91	0.24 (0.94)	0.80	-1.61, 2.08
HbA1c (mmol/mol)	Case	4.42 (0.99)	<0.01	2.49, 6.35	5.75 (1.04)	<0.01	3.72, 7.79
	T2	-0.39 (0.78)	0.62	-1.91, 1.14	1.22 (0.73)	0.10	-0.22, 2.66
	T3	Reference	--	--	Reference	--	--
	T4	1.82 (1.05)	0.08	-0.24, 3.89	3.50 (1.52)	0.02	0.51, 6.48
	T5	2.14 (1.12)	0.06	-0.05, 4.33	3.84 (1.26)	<0.01	1.36, 6.32
	Case#T2	-2.52 (1.08)	-4.64	-4.64, -0.41	-3.98 (1.01)	<0.01	-5.96, -2.01
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	7.28 (1.49)	4.36	4.36, 10.2	6.03 (2.24)	0.01	1.63, 10.4
	Case#T5	8.32 (1.60)	5.18	5.18, 11.5	7.29 (1.83)	<0.01	3.71, 10.9
	Constant	33.7 (3.44)	<0.01	27.0, 40.5	24.1 (3.27)	<0.01	17.7, 30.5
Glucose (mmol/L)	Case	0.98 (0.43)	0.02	0.15, 1.82	0.96 (0.31)	<0.01	0.36, 1.57
	T2	-0.57 (0.28)	0.04	-1.12, -0.02	-0.66 (0.21)	<0.01	-1.07, -0.25
	T3	Reference	--	--	Reference	--	--
	T4	-0.18 (0.31)	0.57	-0.78, 0.43	-0.18 (0.41)	0.67	-0.99, 0.63
	T5	0.24 (0.45)	0.59	-0.64, 1.12	0.18 (0.40)	0.65	-0.60, 0.97
	Case#T2	-0.94 (0.41)	0.02	-1.73, -0.14	-0.49 (0.31)	0.11	-1.10, 0.12
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	0.52 (0.45)	0.24	-0.35, 1.39	0.53 (0.61)	0.39	-0.67, 1.73
	Case#T5	0.55 (0.67)	0.42	-0.77, 1.86	1.43 (0.61)	0.02	0.24, 2.61
	Constant	4.61 (0.80)	<0.01	3.06, 6.17	5.68 (0.62)	<0.01	4.47, 6.89
GGT (U/L)	Case	1.91 (5.40)	0.72	-8.68, 12.5	16.7 (5.99)	0.01	4.95, 28.4
	T1	-1.43 (3.34)	0.67	-7.97, 5.11	-4.82 (3.06)	0.12	-10.8, 1.17
	T2	Reference	--	--	Reference	--	--
	T4	-3.14 (5.76)	0.59	-14.4, 8.16	6.30 (7.11)	0.38	-7.63, 20.2
	Case#T1	-3.72 (3.94)	0.35	-11.5, 4.01	-10.8 (4.20)	0.01	-19.1, -2.61
	Case#T2	Reference	--	--	Reference	--	--
	Case#T4	10.7 (7.06)	0.13	-3.18, 24.5	-3.53 (9.80)	0.72	-22.7, 15.7
	Constant	-46.0 (20.7)	0.03	-86.5, -5.48	-2.05 (12.0)	0.86	-25.6, 21.4

[a]50 cases and 69 controls at T1-T3, 44 cases and 53 controls at T4, 26 cases and 40 controls at T5.

[b]54 cases and 61 controls at T1-T3, 38 cases and 38 controls at T4, 20 cases and 28 controls at T5.

Models adjusted for BMI, age, elevated blood pressure, physical activity, and type 2 diabetes family history.

T: time-point; TSH: thyroid stimulating hormone; GGT: gamma-glutamyltransferase.

Table S4. OR and 95% CI for the associations between pre-diagnostic blood biomarker concentrations and incident type 2 diabetes in females. The Tromsø Study 1986-2016.

Females	**T1 1986/87**		**T2 1994/95**		**T3 2001**	
	Crude	**Adjusted[a]**	**Crude**	**Adjusted[a]**	**Crude**	**Adjusted[a]**
Biomarker	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Total lipids ≥7.40 g/L	2.07 (0.98, 4.34)	1.49 (0.58, 3.82)	3.42 (1.58, 7.40)*	1.86 (0.66, 5.23)	3.56 (1.66, 7.63)*	4.68 (1.66, 13.2)*
Total lipids (g/L)	1.34 (1.03, 1.73)*	1.21 (0.85, 1.73)	1.51 (1.16, 1.97)*	1.49 (1.08, 2.07)*	1.63 (1.19, 2.25)*	1.69 (1.12, 2.54)*
Triglycerides ≥1.70 mmol/L	2.42 (1.05, 5.56)*	1.61 (0.61, 4.29)	3.63 (1.62, 8.13)*	2.82 (1.02, 7.78)*	5.43 (2.41, 12.2)*	4.81 (1.80, 12.8)*
Triglycerides (mmol/L)	2.39 (1.35, 4.24)*	1.77 (0.92, 3.42)	2.56 (1.48, 4.45)*	1.95 (1.00, 3.78)*	3.24 (1.76, 5.95)*	3.02 (1.53, 5.98)*
Total cholesterol ≥5.00 mmol/L	1.58 (0.62, 4.06)	1.07 (0.27, 4.28)	7.35 (0.90, 60.0)	3.99 (0.41, 39.1)	2.01 (0.76, 5.29)	1.87 (0.60, 5.85)
Total Cholesterol (mmol/L)	1.16 (0.84, 1.59)	1.03 (0.64, 1.66)	1.40 (1.02, 1.92)*	1.38 (0.89, 2.16)	1.24 (0.86, 1.80)	1.21 (0.76, 1.95)
HDL ≥1.29 mmol/L	0.33 (0.15, 0.72)*	0.37 (0.16, 0.95)*	0.35 (0.16, 0.76)*	0.51 (0.20, 1.32)	0.30 (0.13, 0.67)*	0.47 (0.18, 1.25)
HDL (mmol/L)	0.30 (0.10, 0.87)*	0.49 (0.14, 1.64)	0.28 (0.10, 0.81)*	0.61 (0.17, 2.21)	0.15 (0.05, 0.45)*	0.21 (0.06, 0.74)*
LDL ≥3.00 mmol/L	1.22 (0.46, 3.20)	0.71 (0.18, 2.83)	1.35 (0.42, 4.30)	0.51 (0.10, 2.56)	1.85 (0.73, 4.68)	1.02 (0.32, 3.27)
LDL (mmol/L)	1.05 (0.74, 1.49)	0.91 (0.55, 1.52)	1.31 (0.93, 1.86)	1.25 (0.76, 2.06)	1.24 (0.84, 1.83)	1.15 (0.70, 1.86)
Free T_3 ≥5.20	1.19 (0.52, 2.71)	0.99 (0.38, 2.57)	NA	NA	3.62 (1.50, 8.72)*	3.30 (1.14, 9.57)*
Free T3 (pmol/L)	1.28 (0.65, 2.52)	1.01 (0.45, 2.28)	NA	NA	2.72 (1.36, 5.46)*	2.39 (1.01, 5.66)*
Free T_4 ≥14.8	0.53 (0.24, 1.13)	0.47 (0.19, 1.14)	NA	NA	0.31 (0.14, 0.70)*	0.26 (0.10, 0.72)*
Free T4 (pmol/L)	0.92 (0.78, 1.09)	0.92 (0.77, 1.11)	NA	NA	0.97 (0.85, 1.11)	0.95 (0.81, 1.12)
TSH ≥1.92	1.53 (0.73, 3.19)	1.14 (0.49, 2.66)	1.41 (0.36, 5.49)	0.19 (0.01, 2.63)	1.25 (0.60, 2.60)	1.03 (0.43, 2.49)
TSH (mIU/L)	0.90 (0.70, 1.16)	0.80 (0.52, 1.22)	0.87 (0.54, 1.39)	0.68 (0.24, 1.95)	0.90 (0.71, 1.15)	0.82 (0.51, 1.32)
HbA1c ≥39.0 mmol/mol	NA	NA	5.08 (1.65, 15.6)*	6.16 (1.42, 26.7)*	14.7 (4.93, 44.0)*	15.7 (4.66, 53.1)*
HbA1c (mmol/mol)	NA	NA	1.14 (1.02, 1.26)*	1.15 (1.00, 1.32)*	1.29 (1.16, 1.43)*	1.31 (1.14, 1.51)*
Glucose ≥5.78 mmol/L	NA	NA	6.81 (1.39, 33.3)*	55.4 (1.26, 187)*	8.48 (3.46, 20.8)*	4.23 (1.49, 12.0)*
Glucose (mmol/L)	NA	NA	5.01 (2.21, 11.4)*	5.37 (1.86, 15.5)*	2.34 (1.47, 3.73)*	1.74 (1.06, 2.84)*
GGT ≥20.0 U/L	3.63 (1.27, 10.4)*	2.77 (0.85, 9.10)	6.43 (2.86, 14.4)*	5.10 (1.95, 13.4)*	NA	NA
GGT (U/L)	1.05 (1.01, 1.09)*	1.04 (0.99, 1.08)	1.03 (1.01, 1.05)*	1.02 (1.00, 1.04)*	NA	NA

[a]Adjusted for age, BMI, physical activity, elevated blood pressure, and family history of type 2 diabetes. TSH: thyroid stimulating hormone; GGT: gamma-glutamyltransferase; *p<0.05

Table S5. OR and 95% CI for the associations between pre-diagnostic blood biomarker concentrations and incident type 2 diabetes in males. The Tromsø Study 1986-2016.

Males	**T1 1986/87**		**T2 1994/95**		**T3 2001**	
	Crude	**Adjusted[a]**	**Crude**	**Adjusted[a]**	**Crude**	**Adjusted[a]**
Biomarker	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**
Total lipids ≥7.59 g/L	3.20 (1.47, 6.93)*	1.99 (0.82, 4.82)	2.05 (0.96, 4.36)	1.26 (0.53, 3.00)	2.81 (1.29, 6.14)*	3.31 (1.37, 7.99)*
Total lipids (g/L)	1.53 (1.16, 2.03)*	1.26 (0.93, 1.72)	1.03 (0.87, 1.22)	0.93 (0.75, 1.16)	1.10 (0.84, 1.44)	0.92 (0.67, 1.27)
Triglycerides ≥1.70 mmol/L	2.88 (1.34, 6.17)*	2.11 (0.88, 5.07)	1.57 (0.73, 3.34)	0.84 (0.34, 2.11)	2.29 (1.08, 4.84)*	1.91 (0.77, 4.75)
Triglycerides (mmol/L)	1.69 (1.11, 2.57)*	1.26 (0.79, 2.01)	1.06 (0.84, 1.34)	0.96 (0.73, 1.26)	1.49 (0.98, 2.26)	1.17 (0.77, 1.77)
Total cholesterol ≥5.00 mmol/L	1.48 (0.53, 4.13)	0.98 (0.29, 3.25)	1.69 (0.53, 5.42)	0.78 (0.19, 3.21)	0.70 (0.29, 1.68)	0.43 (0.15, 1.21)
Total Cholesterol (mmol/L)	1.52 (1.09, 2.12)*	1.28 (0.88, 1.86)	1.00 (0.73, 1.37)	0.83 (0.56, 1.23)	0.83 (0.58, 1.20)	0.70 (0.44, 1.11)
HDL ≥1.03 mmol/L	1.23 (0.59, 2.59)	1.90 (0.78, 4.66)	0.82 (0.38, 1.74)	1.58 (0.63, 3.96)	0.56 (0.25, 1.23)	0.83 (0.33, 2.12)
HDL (mmol/L)	0.49 (0.14, 1.66)	0.82 (0.19, 3.48)	0.65 (0.19, 2.15)	1.33 (0.32, 5.51)	0.28 (0.09, 0.91)*	0.61 (0.16, 2.34)
LDL ≥3.00 mmol/L	0.97 (0.38, 2.50)	0.82 (0.28, 2.40)	1.08 (0.42, 2.74)	0.73 (0.25, 2.14)	0.46 (0.19, 1.14)	0.36 (0.11, 1.18)
LDL (mmol/L)	1.25 (0.91, 1.73)	1.17 (0.81, 1.69)	0.98 (0.68, 1.43)	0.86 (0.54, 1.35)	0.73 (0.50, 1.09)	0.62 (0.38, 1.02)
Free T_3 ≥5.12	1.44 (0.68, 3.05)	1.40 (0.58, 3.37)	NA	NA	2.23 (1.03, 4.84)*	3.09 (1.18, 8.09)*
Free T3 (pmol/L)	1.35 (0.72, 2.56)	1.33 (0.60, 2.98)	NA	NA	1.81 (0.95, 3.45)	2.27 (0.90, 5.76)
Free T_4 ≥14.0	0.54 (0.25, 1.16)	0.84 (0.33, 2.12)	NA	NA	0.56 (0.27, 1.17)	0.77 (0.34, 1.79)
Free T4 (pmol/L)	0.92 (0.78, 1.10)	1.08 (0.86, 1.35)	NA	NA	0.92 (0.79, 1.07)	1.06 (0.87, 1.29)
TSH ≥1.85	1.44 (0.68, 3.05)	1.10 (0.48, 2.53)	0.42 (0.11, 1.60)	0.53 (0.10, 2.87)	1.08 (0.51, 2.27)	1.10 (0.47, 2.56)
TSH (mIU/L)	0.99 (0.70, 1.39)	0.79 (0.52, 1.20)	0.63 (0.32, 1.22)	0.57 (0.22, 1.43)	0.87 (0.63, 1.19)	0.91 (0.62, 1.34)
HbA1c ≥39.0 mmol/mol	NA	NA	4.81 (1.43, 16.3)*	4.59 (1.15, 18.3)*	6.61 (2.70, 16.2)*	8.58 (2.96, 24.9)*
HbA1c (mmol/mol)	NA	NA	1.20 (1.04, 1.37)*	1.15 (0.98, 1.34)*	1.25[a] (1.12, 1.39)*	1.27 (1.11, 1.45)*
Glucose ≥5.59 mmol/L	NA	NA	1.39 (0.43, 4.52)	1.39 (0.35, 5.64)	3.47 (1.61, 7.50)*	3.29 (1.38, 7.81)*
Glucose (mmol/L)	NA	NA	1.35 (0.71, 2.57)	1.15 (0.54, 2.46)	1.98 (1.28, 3.05)*	1.96 (1.19, 3.21)*
GGT ≥25.0 U/L	3.36 (1.51, 7.47)*	2.28 (0.91, 5.71)	4.86 (2.17, 10.9)*	3.13 (1.24, 7.94)*	NA	NA
GGT (U/L)	1.01 (1.00, 1.03)*	1.00 (0.98, 1.02)	1.02 (1.00, 1.04)*	1.00 (0.99, 1.02)	NA	NA

[a]Adjusted for age, BMI, physical activity, elevated blood pressure, and family history of type 2 diabetes. TSH: thyroid stimulating hormone; GGT: gamma-glutamyltransferase; *p<0.05

Paper II

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Longitudinal changes in vitamin D concentrations and the association with type 2 diabetes mellitus: the Tromsø Study.

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ORIGINAL ARTICLE

Longitudinal changes in vitamin D concentrations and the association with type 2 diabetes mellitus: the Tromsø Study

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Abstract

Aim We aimed to investigate the relationship between pre- and post-diagnostic 25-hydroxyvitamin D (25(OH)D) concentrations and type 2 diabetes (T2DM) over a period of 30 years in individuals who developed T2DM compared to healthy controls.

Methods This case–control study included 254 participants with blood samples collected at five different time-points (T1–T5) between 1986 and 2016. Of the 254 participants, 116 were diagnosed with T2DM between T3 and T4, and were considered cases; the remaining 138 were controls. Linear mixed regression models were used to examine pre- and post-diagnostic changes in 25(OH)D concentrations, and logistic regression was used to examine associations between these concentrations and T2DM at each time-point.

Results 25(OH)D concentrations at different time-points and the longitudinal change in concentrations differed between cases and controls, and by sex. For women, each 5-nmol/l increase in 25(OH)D concentrations was inversely associated with T2DM at T3 (odds-ratio, OR, 0.79), whereas for men, this same increase was positively associated with T2DM at T1 (OR 1.12). Cases experienced a significant decrease in pre-diagnostic 25(OH)D concentrations (p value <0.01 for women, p value $=0.02$ for men) and a significant increase in post-diagnostic 25(OH)D concentrations (p value <0.01 for women, p value $=0.01$ for men). As such, each 1-unit increase in month-specific z-score change between T1 and T3 was significantly inversely associated with T2DM (OR 0.51 for women, OR 0.52 for men), and each such increase between T3 and T5 was significantly positively associated with T2DM in women (OR 2.48).

Conclusions 25(OH)D concentrations seem to be affected by disease progression and type 2 diabetes diagnosis.

Keywords 25 Hydroxyvitamin D 3 · 25 Hydroxyvitamin D 2 · Longitudinal survey · Type 2 diabetes mellitus

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Introduction

The prevalence of type 2 diabetes (T2DM) has increased over the past decades, and this increase is projected to continue [1, 2]. As part of an effort to improve the prevention and treatment of T2DM, there has been an increased interest in assessing risk factors as potential targets for interventions; one such risk factor is vitamin D [3, 4]. Vitamin D is metabolised in the liver to 25-hydroxyvitamin D (25(OH)D) and then further metabolised in the kidneys to the biologically active form, 1,25-dihydroxyvitamin D (1,25(OH)2D) [5, 6]. Vitamin D status is mainly based on 25(OH)D concentration, due to its longer half-life; 1,25(OH)2D is not generally used, as it is tightly regulated by the kidneys and levels are often normal in vitamin D-deficient individuals [5, 7]. The main function of vitamin D is to regulate calcium

and phosphate levels in bone metabolism, but may also be involved in glycemic control, beta cell protection, and insulin secretion and resistance as vitamin D receptors are present in pancreatic beta cells and in target tissues for insulin, such as the liver, skeletal muscle, and adipose tissue [8–11].

Longitudinal studies have reported significant associations between vitamin D deficiency and increased risk of T2DM [12, 13]. Repeated measurements of vitamin D in the same individuals who received healthy lifestyle advice demonstrated that improved vitamin D status over time was associated with reduced risk of T2DM over a mean follow-up of 1.1–2.7 years [14, 15]. In contrast, vitamin D supplements have not proven to improve glycaemic control or reduce the risk of T2DM; hence, causality has not been established [16]. Pittas et al. suggests that the difficulties in assessing causality between vitamin D and T2DM might be due to the slow progression, complexity, and heterogeneity of the disease [16]. Accordingly, vitamin D levels are associated with several other risk factors for T2DM, such as age, body weight, and physical activity (as a proxy for sun exposure and energy expenditure); hence, associations between vitamin D and T2DM may be confounded by these risk factors [8, 17]. Repeated measurements yield more accurate measures of exposures and confounders than a single baseline measurement [18], and the Tromsø Study provides a unique opportunity to explore the longitudinal relationship between vitamin D, risk factors, and T2DM, with three to five repeated measurements for every participant. The present study aimed to investigate the relationship between pre- and post-diagnostic 25(OH)D concentrations and T2DM over a period of 30 years in individuals who developed T2DM compared to healthy controls.

Materials and methods

Study population

The Tromsø Study is an ongoing health survey based on the residents of the municipality of Tromsø in Northern Norway [19, 20]. Briefly, it was initiated in 1974, with surveys conducted approximately every 7 years; to-date, seven surveys have been completed (Tromsø1 through Tromsø7). At each survey, participants answered questionnaires, attended physical examinations, and had blood samples collected, which were frozen and stored as serum at − 70 °C.

We used a longitudinal nested case–control design with repeated measurements from Tromsø3 (1986/87), Tromsø4 (1994/95), Tromsø5 (2001), Tromsø6 (2007/08), and Tromsø7 (2015/16), which we will refer to as time-points 1 through 5 (T1 through T5). The inclusion criteria for cases were T2DM diagnosis recorded in the local diabetes registry after the year 2000 (between T3 and T4), and available pre-diagnostic serum samples at T1, T2, and T3. Seventy-six women and 69 men met these criteria. Controls were randomly selected among those who had no T2DM diagnosis recorded in a local diabetes registry and then matched 1:1 by sex and participation in the same surveys as cases. In total, 290 participants were eligible for inclusion for T1–T3, of which 130 attended T4 and 122 attended T5 and had available serum samples. We excluded 29 cases with glycated haemoglobin (HbA_{1c}) levels higher than 48 mmol/mol (6.5%) at T3 or earlier, and seven controls with HbA_{1c} levels higher than 48 mmol/mol at any time-point. The final sample included 254 participants at T1, T2, and T3, respectively, 119 at T4, and 108 at T5 (989 serum samples in total, Fig. 1). Informed consent was received at each survey from all the participants. The Regional Ethics Committee, REK, Nord approved the study protocol (REK reference: 2015/1780/REK Nord).

Vitamin D analysis

Serum samples were randomised in batches within each time-point with equal amounts of cases, controls, men, and women, and were thawed and analysed for total 25(OH)D (hereafter referred to as 25(OH)D) over a period of 2 weeks at the Department of Laboratory Medicine, University Hospital of North Norway. Laboratory technicians were blinded to the sample number and time-point. The laboratory is a clinical laboratory accredited by the ISO 15189 standard and routinely runs vitamin D testing by liquid–liquid extraction (LLE) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) detection, as described in detail elsewhere [21]. LLE was performed on a Tecan Fluent liquid handler (Männedorf, Switzerland), and LC–MS/MS detection was performed on a Waters Acquity™ *I*-class (Waters, Milford, MA) interfaced with Waters Xevo TQ-XS (Waters, Manchester, UK). MassCheck® quality control levels 1 and 2 for 25(OH)D (Chromsystems Instruments & Chemicals GmbH, München, Germany) were included with each batch, and the controls deviated less than 5% from the target values. The laboratory participates in an external proficiency programme (DEQAS, UK) and performs well within accepted target range values.

Statistical analyses

25(OH)D concentrations and sample characteristics are reported as means with standard deviation (SD), and/or frequencies with percentages. Sample characteristics were compared between cases and controls at each time-point using independent two-sample *t* tests for continuous variables and Pearson's χ^2 test for categorical variables.

Potential confounding variables in the causal pathway between 25(OH)D and T2DM were identified by a directed

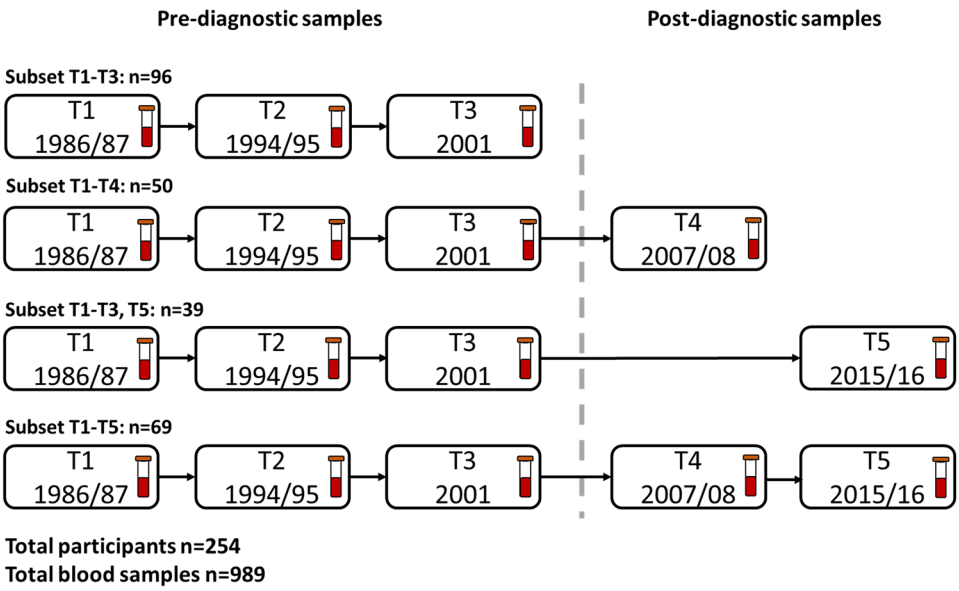

Fig. 1 Overview of available serum samples and subsets based on participation in different surveys (time-points, T) of the Tromsø Study 1986–2016. The stippled line represents the separation between pre- and post-T2DM diagnostic time-points in cases

acyclic graph (DAG, Fig. S1) [22], which indicated that the relationship could be confounded by age, body mass index (BMI), weight change between time-points (T1 set to zero), and physical activity (active: ≥ 3 h/week of light activity and/or ≥ 1 h hard exercise/week; inactive: < 3 h/week of activity that provoked perspiration or no activity). Month of blood sample collection (as a proxy for exposure to sunlight) and cod liver oil intake were not identified as confounders in the DAG. However, these variables varied by case–control status and time-point, and could have affected the time trends in 25(OH)D concentrations. Therefore, we added these two variables in the adjustment of time-trend analyses.

Linear mixed effects models were used to examine changes in 25(OH)D from T1 to T5, between and within cases and controls, after adjusting for DAG confounders, month of blood sample collection, and cod liver oil intake. 25(OH)D concentration (continuous) was used as the dependent variable; T2DM status, DAG confounders, month of blood sample collection, cod liver oil intake, and indicator variables of time with two-way interaction terms with T2DM status were used as independent variables. A random intercept at the participant level to control for repeated measurements over time, and an unstructured variance and covariance correlation structure for within-group errors was used. To fully explore the effect of month of blood collection, we repeated the same model, using month-specific 25(OH)D z-score as a dependent variable and removing month of blood collection as a confounder.

We used logistic regression to estimate odds-ratios (OR) for the association between 25(OH)D and T2DM at each time-point. We applied models with 25(OH)D as a continuous and dichotomised (< 50 nmol/l, i.e. vitamin D deficient and ≥ 50 nmol/l, i.e. vitamin D sufficient) independent variables, with T2DM status as the dependent variable, and adjusted for DAG confounders. To take advantage of the repeated measurements, we further calculated the area under the curve (AUC) for month-specific 25(OH)D z-score (to account for the variation in the month of blood sample collection between time-points) for pre-diagnostic samples. The AUC was then used as the independent variable in a logistic regression model along with DAG confounders measured at T1. Similarly, to explore associations between changes in 25(OH)D concentrations and T2DM in logistic regression models, we included the difference in month-specific 25(OH)D z-score (Δ25(OH)D) for each individual between T1 and T3, and between T3 and T5 as independent variables along with DAG confounders from T1 and T3, respectively.

Statistical analyses were performed in STATA (v 17.0, StataCorp LLC, 4905 Lakeway Drive, College Station, Texas USA). Significance was set at 5%, and p values were two-sided. All analyses were stratified by sex.

Results

Study sample characteristics

Cases and controls were similar in age and experienced the same weight change throughout the study period (Table 1). Cases were significantly heavier and had higher BMI at all time-points. Cases and controls had a similar physical activity level and cod liver oil intake, except for women at T2 and T5, and men at T5, where controls were more active, and at T3, where women controls had higher cod liver oil intake. Month of blood sample collection was similar for cases and controls at each time-point (p values 0.11–0.97), but varied

Table 1 Selected characteristics of cases and controls by time-point. The Tromsø Study 1986–2016

	T1 (1986/87)		T2 (1994/95)		T3 (2001)		T4 (2007/08)		T5 (2015/16	
	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)
Age (years)										
Women										
Case	46.3 (6.36)	2.50 (−0.19, 5.19)	54.3 (6.36)	2.50 (−0.19, 5.19)	61.3 (6.36)	2.50 (−0.19, 5.19)	66.8 (6.55)	1.14 (−1.58, 3.87)	74.1 (6.14)	3.55 (−0.38, 7.49)
Control	43.8 (8.88)		51.8 (8.88)		58.8 (8.88)		65.7 (7.71)		70.5 (9.47)	
Men										
Case	48.8 (8.66)	2.09 (−1.49, 5.64)	56.8 (8.66)	2.09 (−1.49, 5.64)	63.8 (8.66)	2.09 (−1.49, 5.64)	69.1 (7.22)	1.92 (−2.01, 5.85)	73.3 (8.42)	3.12 (−2.66, 8.90)
Control	46.7 (10.7)		54.7 (10.7)		61.7 (10.7)		67.2 (10.1)		70.2 (11.0)	
BMI (kg/m^2)										
Women										
Case	27.1 (4.27)	3.35** (1.97, 4.72)	29.2 (4.91)	4.21** (2.58, 5.83)	31.2 (5.69)	4.88** (3.12, 6.63)	31.3 (6.09)	4.66** (2.54, 6.78)	31.4 (6.85)	4.56* (1.59, 7.53)
Control	23.7 (3.75)		25.0 (4.61)		26.4 (4.60)		26.6 (5.04)		26.8 (5.76)	
Men										
Case	27.6 (3.49)	2.92** (1.79, 4.05)	28.7 (3.44)	3.01** (1.84, 4.18)	29.8 (3.52)	3.28** (2.02, 4.55)	29.5 (3.38)	2.51** (1.01, 4.00)	29.4 (3.74)	2.20* (0.11, 4.29)
Control	24.7 (2.72)		25.7 (3.02)		26.6 (3.44)		26.9 (3.25)		27.2 (3.49)	
Weight (kg)										
Women										
Case	70.8 (11.6)	7.95** (4.28, 11.6)	76.2 (13.1)	10.2** (5.96, 14.4)	80.6 (14.5)	11.5** (6.97, 16.0)	80.6 (15.1)	11.9** (6.55, 17.2)	81.3 (17.3)	11.8** (4.15, 19.5)
Control	62.9 (9.89)		66.0 (11.7)		69.1 (12.0)		68.8 (13.0)		69.4 (15.2)	
Men										
Case	85.5 (12.9)	7.11** (3.08, 11.1)	88.8 (13.3)	7.66** (3.41, 11.9)	91.6 (13.8)	8.01** (3.39, 12.6)	90.7 (11.8)	5.94* (0.84, 11.0)	89.7 (14.3)	3.71 (−3.74, 11.2)
Control	78.3 (9.16)		81.2 (10.1)		83.6 (11.6)		84.8 (10.8)		86.0 (11.7)	
Weight change (kg)										
Women										
Case	NA	NA	5.36 (4.34)	2.25* (0.68, 3.82)	4.44 (5.47)	1.30 (−0.70, 3.29)	−0.57 (5.77)	−1.12 (−3.24, 0.99)	−1.55 (5.93)	−1.38 (−4.12, 1.37)
Control	NA	NA	3.11 (4.73)		3.14 (6.04)		0.55 (5.39)		-0.17 (5.15)	
Men										
Case	NA	NA	3.38 (4.07)	0.55 (−1.04, 2.14)	2.79 (5.20)	0.34 (−1.66, 2.35)	−2.46 (6.10)	−2.44 (−5.02, 0.15)	−1.05 (8.26)	−1.98 (−6.36, 2.40)
Control	NA	NA	2.83 (4.64)		2.44 (5.78)		−0.03 (5.34)		0.93 (6.12)	
25(OH)D concentration (nmol/l)										
Women										
Case	44.8 (12.6)	−1.18 (−5.99, 3.62)	53.6 (14.9)	−2.85 (−7.88, 2.18)	46.6 (13.6)	−13.5** (−19.3, −7.72)	50.9 (11.5)	−11.4* (−21.6, -1.34)	69.7 (24.6)	1.06 (−11.5, 13.6)
Control	46.0 (15.1)		56.4 (14.5)		60.1 (19.1)		62.3 (27.9)		68.6 (24.6)	

Table 1 (continued)

	T1 (1986/87)		T2 (1994/95)		T3 (2001)		T4 (2007/08)		T5 (2015/16	
	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)	Mean (SD)	ΔMean case–control (95% CI)
Men										
Case	53.4 (17.1)	6.46* (0.04, 12.6)	58.5 (16.4)	2.39 (−3.28, 8.07)	53.3 (13.6)	−5.22 (−10.8, 0.32)	59.7 (26.5)	7.42 (−5.96, 20.8)	67.5 (22.6)	8.73 (−3.70, 21.2)
Control	47.0 (16.5)		56.1 (14.8)		58.5 (16.5)		52.3 (15.9)		58.8 (16.2)	
	n (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value
Physical activity: women										
Active										
Case	48 (80.0)	0.71	26 (43.3)	0.004	44 (74.6)	0.64	33 (73.3)	0.07	19 (73.1)	0.05
Control	58 (77.3)		51 (68.0)		57 (78.1)		44 (88.0)		33 (91.7)	
Inactive										
Case	12 (20.0)		34 (56.7)		15 (25.4)		12 (26.7)		7 (26.9)	
Control	17 (22.7)		24 (32.0)		16 (21.9)		6 (12.0)		3 (8.3)	
Physical activity: men										
Active										
Case	46 (82.1)	0.96	39 (69.6)	0.55	42 (77.8)	0.62	28 (71.8)	0.51	9 (45.0)	<0.001
Control	52 (82.5)		47 (74.6)		45 (73.8)		29 (78.4)		24 (92.3)	
Inactive										
Case	10 (17.9)		17 (30.4)		12 (22.2)		11 (28.2)		11 (55.0)	
Control	11 (17.5)		16 (25.4)		16 (26.2)		8 (21.6)		2 (7.7)	
Cod liver oil intake: women										
No										
Case	40 (81.6)	0.16	29 (58.0)	0.21	25 (44.6)	0.01	23 (54.8)	0.96	19 (67.9)	0.08
Control	47 (70.2)		29 (46.0)		17 (23.3)		26 (55.3)		16 (45.7)	
Yes										
Case	9 (18.4)		21 (42.0)		31 (55.4)		19 (45.2)		9 (32.1)	
Control	20 (29.9)		34 (54.0)		56 (76.7)		21 (44.7)		19 (54.3)	
Cod liver oil intake: men										
No										
Case	33 (71.7)	0.98	26 (50.0)	0.86	26 (54.2)	0.1	17 (50.0)	0.37	14 (73.7)	0.25
Control	41 (71.9)		30 (51.7)		23 (38.3)		23 (60.5)		16 (57.1)	
Yes										
Case	13 (28.3)		26 (50.0)		22 (45.8)		17 (50.0)		5 (26.3)	
Control	16 (28.1)		28 (48.3)		37 (61.7)		15 (39.5)		12 (42.9)	

Table 1 (continued)

	n (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value	*n* (%)	*p* value
Vitamin D status (nmol/l): women										
>50										
Case	21 (35.0)	0.23	32 (53.3)	0.21	21 (35.0)	<0.001	17 (48.6)	0.02	21 (72.4)	0.09
Control	34 (45.3)		48 (64.0)		51 (68.9)		29 (74.4)		25 (75.8)	
<50										
Case	39 (65.0)		28 (46.7)		39 (65.0)		18 (51.4)		8 (27.6)	
Control	41 (54.7)		27 (36.0)		23 (31.1)		10 (25.6)		8 (24.2)	
Vitamin D status (nmol/l): men										
>50										
Case	33 (58.9)	0.02	39 (69.6)	0.98	30 (53.6)	0.15	15 (71.4)	0.15	15 (83.3)	0.27
Control	24 (38.1)		44 (69.8)		42 (66.7)		11 (50.0)		15 (68.2)	
<50										
Case	23 (41.1)		17 (30.4)		26 (46.4)		6 (28.6)		3 (16.7)	
Control	39 (61.9)		19 (30.2)		21 (33.3)		11 (50.0)		7 (31.8)	

Weight change was calculated between time-points (T1 set to zero). Pre-diagnostic period: T1 to T3. Post-diagnostic period: T4 to T5

25(OH)D 25-hydroxyvitamin D, *CI* confidence interval, *SD* standard deviation, *T* time-point

*p value <0.05, **p value <0.01

between time-points (p values < 0.01). At T1, more blood samples were collected from December to February and from September to November, whereas at other time-points, blood sample collection was distributed more evenly across the year (Table S1). At T2, only 10 blood samples were collected from June to August, and at T3, only four blood samples were collected from December to February.

Vitamin D concentrations

In women, cases had lower 25(OH)D concentrations than controls at every time-point (significantly at T3 and T4) except T5, when concentrations were similar (Table 1). In men, cases had higher 25(OH)D concentrations than controls at all time-points (significantly at T1) except T3, when concentrations were lower in cases. Among women, there was a significantly higher percentage of cases than controls with insufficient vitamin D status at T3 and T4. For men, there was a significantly higher percentage of controls with insufficient vitamin D status at T1 (Table 1).

Longitudinal changes in vitamin D

After adjusting for DAG confounders (age, BMI, weight change, physical activity), month of blood sample collection and cod liver oil intake, all participants' 25(OH)D concentrations increased from T1 to T2, followed by a decrease from T2 to T3 (Fig. 2 and Table S2). Cases experienced a significantly larger decrease in 25(OH)D concentrations from T2 to T3 compared to controls. Further, post-diagnostic (T3 to T5) 25(OH)D concentrations increased in cases compared to controls; the latter experienced an overall decrease. Repeating the analyses using month-specific 25(OH)D z-scores yielded similar results (results not presented).

Associations between vitamin D and T2DM

At T1, a 5-nmol/l increase in 25(OH)D concentration was associated with 15% higher odds (OR 1.15, 95% CI 1.00, 1.31) for T2DM in men. Likewise, sufficient vitamin D status was positively associated with T2DM compared to insufficient vitamin D status (OR 2.98, 95% CI 1.24, 7.17). In women, a 5-nmol/l increase in 25(OH)D concentration was associated with 21% lower odds of T2DM (OR 0.79, 95% CI 0.68, 0.91) at T3. At the same time-point, sufficient vitamin D status was inversely associated with T2DM compared to insufficient vitamin D status (OR 0.29, 95% CI 0.13, 0.69) (Table 2). At all other time-points, neither 25(OH)D concentrations nor vitamin D status was significantly associated with T2DM. Results were similar when repeating the analyses with month-specific 25(OH)D z-scores (results not presented).

Each 1-unit increase in the pre-diagnostic difference (T3 to T1) in month-specific Δ25(OH)D z-score was significantly and inversely associated with T2DM in both sexes, whereas each 1-unit increase in post-diagnostic difference (T5 to T4) was significantly associated with higher odds of T2DM in women (Table 3). There were no significant associations between pre-diagnostic AUC for 25(OH)D z-score and T2DM.

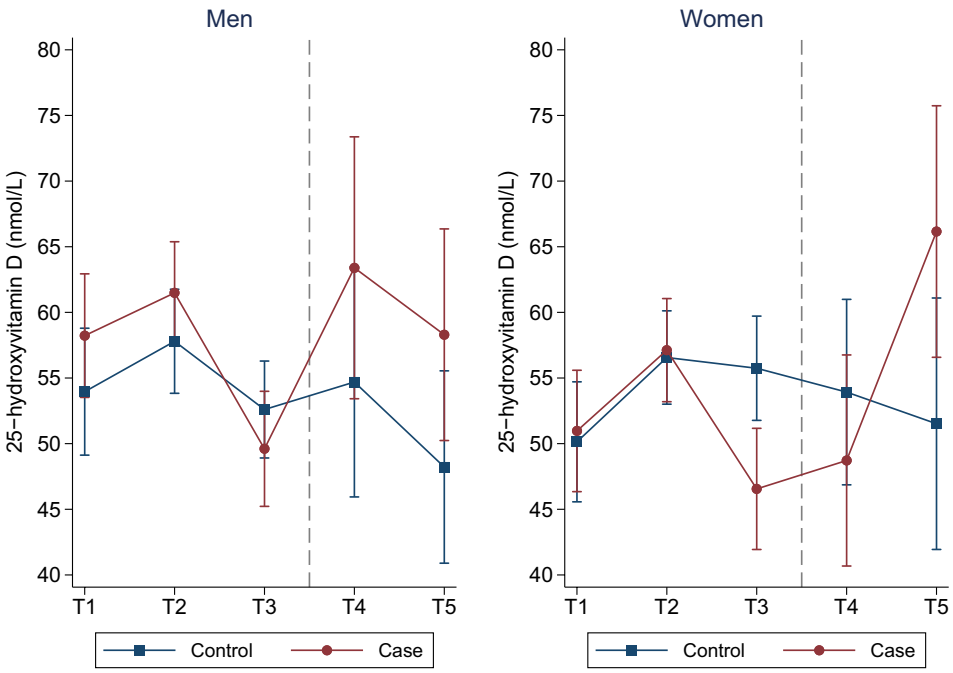

Fig. 2 Estimated mean 25-hydroxyvitamin D concentrations (y-axis) across five time-points for cases and controls. Models were adjusted for age, BMI, weight change, physical activity, month of blood sample collection, and cod liver intake. The Tromsø Study 1986–2016. *T* time-point. Dots/squares represent mean concentrations and whiskers the 95% confidence interval around the mean

Table 2 ORs with 95% CIs for the associations between 25(OH)D concentrations and T2DM in women and men. The Tromsø Study 1986–2016

		T1 (1986/87)		T2 (1994/95)		T3 (2001)		T4 (2007/08)		T5 (2015/16)	
		Crude	Adjusted[a]	Crude	Adjusted[a]	Crude	Adjusted[a]	Crude	Adjusted[a]	Crude	Adjusted[a]
Biomarker	Sex	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)	OR (95% CI)
25(OH)D (per 5-nmol/l increase)	Women	0.97 (0.86, 1.10)	1.00 (0.87, 1.15)	0.93 (0.83, 1.05)	0.97 (0.84, 1.12)	0.77* (0.68, 0.87)	0.79* (0.68, 0.91)	0.79* (0.65, 0.96)	0.79 (0.60, 1.02)	1.01 (0.91, 1.12)	1.06 (0.93, 1.22)
	Men	1.12* (1.00, 1.26)	1.15* (1.00, 1.31)	1.05 (0.94, 1.18)	1.09 (0.95, 1.26)	0.89 (0.79, 1.01)	0.90 (0.76, 1.07)	1.09 (0.93, 1.28)	1.14 (0.96, 1.35)	1.13 (0.95, 1.35)	1.21 (0.89, 1.65)
25(OH)D (> 50 nmol/l)[b]	Women	0.65 (0.32, 1.31)	0.65 (0.30, 1.40)	0.64 (0.32, 1.28)	0.75 (0.35, 1.70)	0.24* (0.12, 0.50)	0.29* (0.13, 0.69)	0.33* (0.12, 0.87)	0.25 (0.06, 1.03)	1.84 (0.27, 2.62)	1.03 (0.21, 5.05)
	Men	2.33* (1.12, 4.87)	2.98* (1.24, 7.17)	0.99 (0.45, 2.17)	1.23 (0.46, 3.25)	0.58 (0.27, 1.21)	0.86 (0.32, 2.30)	2.50 (0.71, 8.84)	3.83 (0.75, 19.4)	2.33 (0.51, 10.8)	23.4 (0.74, 743)

25(OH)D 25-hydroxyvitamin D, *CI* confidence interval, *OR* odds-ratio, *T* time-point

*p value < 0.05

[a]Adjusted for age, body mass index, weight change, and physical activity

[b]< 50 nmol/l is set as the reference

Discussion

This is the first observational study with repeated pre- and post-diagnostic 25(OH)D concentrations in T2DM cases and controls over a 30-year time period. Our results suggest that there is an association between changes in 25(OH)D concentrations and T2DM. This is supported by our findings that: (1) cases and controls had similar 25(OH)D concentrations (higher for cases at T1 for men) 7–15 years prior to diagnosis; (2) cases experienced significantly larger pre-diagnostic declines closer to the time of diagnosis, and (3) cases had substantial post-diagnostic increases in 25(OH)D concentrations compared to controls. As a result, decreases in 25(OH)D concentrations in the pre-diagnostic period were inversely associated with T2DM, whereas increases in the post-diagnostic period were positively associated with T2DM. It is likely that pre-diagnostic 25(OH)D concentrations are affected by factors related to disease progression and dietary habits, whereas post-diagnostic concentrations could be impacted by an overall improvement in health following T2DM diagnosis (e.g. dietary counselling and medication). This is supported by our previous findings in this population, where cases significantly improved their lipid profiles after diagnosis [23].

Mendelian randomisation studies and intervention studies have addressed the causal relationship between vitamin D and T2DM but with inconclusive and/or non-significant results [12, 16, 24, 25]. Rejnmark et al. [26] summarised findings from observational studies and concluded that the progression of a large number of diseases, including T2DM, is associated with low vitamin D concentrations; however, intervention studies of vitamin D supplementation on these diseases did not provide causal evidence. A common denominator for diseases associated with low vitamin D concentrations is underlying inflammation [27]. Palaniswami et al. [28] observed a significant association between low vitamin D status and inflammation; however, they reported that neither their Mendelian randomisation analysis nor their review of randomised controlled trials (RCTs) supported a causal relationship. Likewise, a review article by Cannell et al. [29] summarised evidence from RCTs and concluded that several studies reported reduced inflammation with higher vitamin D status. Still, it is not clear if vitamin D can lower inflammation or if inflammation can lower vitamin D. Clearly, the relationship between vitamin D, inflammation, and T2DM is complex, and the order of events prior to disease diagnosis is unclear. Nevertheless, studies comparing vitamin D supplementation vs placebo have consistently reported non-significant risk reductions for T2DM in the vitamin D supplement group, which prompts the use of vitamin D supplements

Table 3 ORs with 95% CIs for the associations between month-specific 25(OH)D *z*-score (as summary variable) and T2DM in women and men. The Tromsø Study 1986–2016

	T1–T3				T3–T5	
	Δ25(OH)D[a]		AUC 25(OH)D[b]		Δ25(OH)D[c]	
	Crude OR (95% CI)	Adjusted[d] OR (95% CI)	Crude OR (95% CI)	Adjusted[d] OR (95% CI)	Crude OR (95% CI)	Adjusted[e] OR (95% CI)
Women	0.45* (0.29, 0.70)	0.51* (0.32, 0.80)	0.80 (0.63, 1.02)	0.85 (0.64, 1.12)	2.21* (1.37, 3.56)	2.48* (1.39, 4.43)
Men	0.48* (0.31, 0.74)	0.52* (0.33, 0.84)	1.07 (0.85, 1.34)	1.23 (0.88, 1.57)	2.09* (1.05, 4.18)	1.93 (0.90, 4.12)

25(OH)D 25-hydroxyvitamin D, *CI* confidence interval, *OR* odds-ratio, *T* time-point

*p value < 0.05

[a]Change in month-specific 25(OH)D *z*-score from T1 to T3

[b]AUC for month-specific 25(OH)D *z*-scores for pre-diagnostic samples

[c]Change in month-specific 25(OH)D *z*-score from T3 to T5

[d]Adjusted for age, body mass index, weight change, and physical activity at T1

[e]Adjusted for age, body mass index, weight change, and physical activity at T3

in individuals at high risk for T2DM [16].The potential benefits of vitamin D supplementation are supported by Lemieux et al. [30] in an intervention study that showed significant improvements in insulin sensitivity and beta-cell function for individuals at high risk of T2DM or had newly diagnosed T2DM.

Our study showed that the associations between 25(OH)D concentrations and T2DM were different in men and women. Around 15 years prior to diagnosis, a positive association between 25(OH)D concentrations and T2DM was observed in men, whereas in women, 25(OH)D concentrations were inversely associated with T2DM at all pre-diagnostic time-points, although they were only significant at the time-point closest to diagnosis (T3) in cases. Wierzbicka et al. [6] discusses several sex-specific factors that may influence vitamin D status differently in men and women, of which per cent body fat and sex hormones play a role in circulating vitamin D levels. They noted that higher testosterone and oestrogens levels in men and women, respectively, were significantly associated with higher vitamin D levels, and that women, who generally have a higher percentage of body fat than men, often have lower circulating vitamin D levels than men. In line with our findings, Schöttker et al. [31] found a significant association between low vitamin D status in women and increased risk of T2DM. Further, most studies observed either an increased risk of T2DM with lower 25(OH)D concentrations [13, 15, 25, 32–36], or non-significant associations [37–40]. To our knowledge, no previous studies have reported positive associations between 25(OH)D concentrations and T2DM.

Inconsistencies across studies could be explained by the complexity of the relationship between vitamin D and T2DM, the slow progression and heterogeneity of the disease, and different follow-up times. This clearly emphasises the importance of repeated measurements that capture variations in 25(OH)D concentrations over time. There are three other studies that included repeated measurements, and, like us, they observed that decreased vitamin D concentrations in the pre-diagnostic period was associated with increased risk of T2DM [14, 15, 41]. The variability in vitamin D concentrations from one time-point to another has been investigated previously in the Tromsø Study. Kubiak et al. [42] reported 25(OH)D concentrations from three time-points over a 21-year period and observed a decrease in the correlation between 25(OH)D concentrations in the same individuals over time. They also identified that change in cod liver oil/vitamin D supplement intake and BMI were important factors for changes in vitamin D status between time-points. As 25(OH)D concentrations are affected by lifestyle habits, which may change greatly throughout an individual's lifetime, a design with repeated measurements from prospective T2DM cases and controls will yield more accurate conclusions about vitamin D and T2DM than studies relying on blood samples collected at one point in time. Accordingly, a major strength of this study is its design, with up to five repeated measurements in cases and controls over a period of 30 years. T2DM diagnosis was ascertained in local registries, and laboratory data and medical records confirmed the absence of T2DM among controls. All 25(OH)D measurements were analysed from thawed serum by LC–MS/MS using accredited standards. However, the observational nature of this study does not allow for causal inference and the precision of our estimates might have been affected by stratifying by sex. We also had fewer blood samples at post-diagnostic time-points, which further affects the precision of estimates at T4 and T5. T2DM diagnosis did not vary over time, but was set at T3 for all cases, which meant we were unable to fully integrate the longitudinal relationship in the logistic regression models [43].

We believe that the generalisability of our results to other populations improves by adjusting for proper confounders that are specific for the Northern Norwegian population such as seasonal variation in sun exposure and dietary intake of cod liver oil and vitamin D supplements, as increased intake of vitamin D from these sources during the winter months reduces the effect of season. Hence, vitamin D concentrations in Norway do not fluctuate by season as much as they do in countries located further south [42, 44, 45].

Conclusion

Our results indicate that pre-diagnostic decreases in vitamin D concentrations are associated with T2DM progression and diagnosis, whereas post-diagnostic increases in concentrations are influenced by intervention and treatment efforts.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00592-022-02001-y.

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Author contribution VB and CR conceived and designed the study. GA and VB contributed to acquiring the data. GA and OMF analysed the data. GA, VB, and CR contributed to the interpretation of the data and wrote the manuscript. TW, MB, and RJ contributed to the interpretation of data and results, and to the revision of the manuscript. OMF and MA contributed to the laboratory and clinical aspects while drafting and revising the manuscript.

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Data availability The dataset in this study, which is not publicly available, was acquired from the Tromsø Study. It may be accessed through an application to the Tromsø Study (https://uit.no/research/tromsostudy).

Declaration

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Human and Animal Rights disclosure Informed consent was received at each survey from all the participants. The Regional Ethics Committee, REK, Nord approved the study protocol (REK reference: 2015/1780/REK Nord).

Informed consent disclosure This research did not involve the use of animals.

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Supplemental material

Longitudinal changes in vitamin D concentrations and the association with type 2 diabetes mellitus – The Tromsø Study

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Table S1. Month of blood sample collection in women and men by time-point. The Tromsø Study 1986-2016.

			Pre-diagnostic time-points			Post-diagnostic time-points	
			T1 (1986/87)	**T2 (1994/95)**	**T3 (2001)**	**T4 (2007/08)**	**T5 (2015/16)**
			n (%)	n (%)	n (%)	n (%)	n (%)
Month of blood sample collection: Women	January	Case	11 (18.3)	11 (18.3)	NA	7 (13.5)	1 (3.3)
		Control	17 (22.7)	14 (18.7)	NA	6 (10.5)	5 (11.9)
	February	Case	8 (13.3)	6 (10.0)	NA	3 (5.8)	1 (3.3)
		Control	12 (16.0)	2 (2.7)	NA	4 (7.0)	4 (9.5)
	March	Case	10 (16.7)	6 (10.0)	5 (8.3)	4 (7.7)	0 (0.0)
		Control	7 (9.3)	10 (13.3)	5 (6.7)	8 (14.0)	5 (11.9)
	April	Case	0 (0.0)	4 (6.7)	4 (6.7)	4 (7.7)	3 (10.0)
		Control	3 (4.0)	7 (9.3)	13 (17.3)	6 (10.5)	2 (4.8)
	May	Case	NA	7 (11.7)	8 (13.3)	3 (5.8)	4 (13.3)
		Control	NA	10 (13.3)	10 (13.3)	3 (5.3)	6 (14.3)
	June	Case	NA	1 (1.7)	8 (13.3)	7 (13.5)	5 (16.7)
		Control	NA	5 (6.7)	9 (12.0)	2 (3.5)	5 (11.9)
	July	Case	NA	NA	NA	NA	NA
		Control	NA	NA	NA	NA	NA
	August	Case	1 (1.7)	NA	2 (3.3)	5 (9.6)	2 (6.7)
		Control	0 (0.0)	NA	8 (10.7)	4 (7.0)	5 (11.9)
	September	Case	5 (8.3)	4 (6.7)	9 (15.0)	8 (15.4)	5 (16.7)
		Control	6 (8.0)	6 (8.0)	5 (6.7)	9 (15.8)	5 (11.9)
	October	Case	11 (18.3)	6 (10.0)	13 (21.7)	7 (13.5)	2 (6.7)
		Control	12 (16.0)	2 (2.7)	16 (21.3)	6 (10.5)	2 (4.8)
	November	Case	10 (16.7)	7 (11.7)	11 (18.3)	0 (0.0)	5 (16.7)
		Control	11 (14.7)	15 (20.0)	6 (8.0)	8 (14.0)	1 (2.4)
	December	Case	4 (6.7)	8 (13.3)	0 (0.0)	4 (7.7)	2 (6.7)
		Control	7 (9.3)	4 (5.3)	3 (4.0)	1 (1.8)	2 (4.8)
Month of blood sample collection: Men	January	Case	13 (23.2)	9 (16.1)	NA	2 (5.0)	0 (0.0)
		Control	11 (17.5)	19 (30.2)	NA	4 (10.3)	2 (7.1)
	February	Case	11 (19.6)	7 (12.5)	NA	1 (2.5)	1 (4.8)
		Control	8 (12.7)	8 (12.7)	NA	2 (5.13)	2 (7.1)
	March	Case	7 (12.5)	5 (8.99	4 (7.1)	5 (12.5)	1 (4.8)
		Control	14 (22.2)	2 (3.2)	3 (4.8)	3 (7.7)	3 (10.7)
	April	Case	2 (3.6)	4 (7.14)	6 (10.7)	4 (10.0)	2 (9.5)
		Control	3 (4.8)	8 (12.7)	3 (4.8)	3 (7.7)	1 (3.6)
	May	Case	NA	9 (16.1)	14 (25.0)	2 (5.0)	3 (14.3)
		Control	NA	6 (9.5)	9 (14.3)	3 (7.7)	2 (7.1)
	June	Case	NA	0 (0.0)	5 (8.9)	4 (10.0)	1 (4.8)
		Control	NA	4 (6.4)	10 (15.9)	4 (10.3)	2 (7.1)
	July	Case	NA	NA	NA	NA	2 (9.5)
		Control	NA	NA	NA	NA	1 (3.6)
	August	Case	0 (0.0)	NA	3 (5.4)	9 (22.5)	1 (4.8)
		Control	1 (1.6)	NA	3 (4.8)	6 (15.4)	3 (10.7)
	September	Case	6 (10.7)	8 (14.3)	7 (12.5)	2 (5.0)	3 (14.3)
		Control	4 (6.4)	4 (6.4)	13 (20.6)	3 (7.7)	3 (10.7)
	October	Case	8 (14.3)	4 (7.14)	11 (19.6)	2 (5.0)	1 (4.8)
		Control	5 (7.9)	4 (6.4)	8 (12.7)	3 (7.7)	5 (17.9)
	November	Case	6 (10.7)	6 (10.7)	6 (10.7)	4 (10.0)	5 (23.8)
		Control	8 (12.7)	2 (3.2)	13 (20.6)	5 (12.8)	4 (14.3)
	December	Case	3 (5.4)	4 (7.14)	0 (0.0)	5 (12.5)	1 (4.8)
		Control	9 (14.3)	6 (9.5)	1 (1.6)	3 (7.7)	0 (0.0)

T, time-point.

Table S2. Linear mixed effects models adjusted for age, BMI, weight change, physical activity, month of blood sample collection, and cod liver oil intake. T3 is set as the reference time-point.

Biomarker		Men			Women		
		β-coefficient (SE)	p-value	95% confidence interval	β-coefficient (SE)	p-value	95% confidence interval
Total 25(OH)D (nmol/l)	Case	-3.00 (2.79)	0.28	-8.47, 2.48	-9.19 (3.03)	<0.01	-15.1, -3.25
	T1	1.36 (2.83)	0.63	-4.19, 6.91	-5.61 (2.73)	0.04	-11.0, -0.25
	T2	5.20 (2.27)	0.02	0.75, 9.65	0.82 (2.21)	0.71	-3.50, 5.14
	T3	Reference	--	--	Reference	--	--
	T4	2.10 (4.32)	0.63	-6.37, 10.6	-1.82 (3.13)	0.56	-7.96, 4.32
	T5	-4.38 (3.66)	0.23	-11.5, 2.79	-4.23 (4.64)	0.36	-13.3, 4.86
	Case#T1	7.27 (3.07)	0.02	1.25, 13.3	10.0 (2.72)	<0.01	4.68, 15.4
	Case#T2	6.68 (2.84)	0.02	1.12, 12.3	9.75 (2.94)	<0.01	3.99, 15.5
	Case#T3	Reference	--	--	Reference	--	--
	Case#T4	11.7 (6.25)	0.06	-0.56, 23.9	3.98 (4.37)	0.36	-4.58, 12.5
	Case#T5	13.1 (4.95)	0.01	3.37, 22.8	23.8 (5.83)	<0.01	12.4, 35.3
	Constant	28.7 (11.5)	0.01	6.10, 51.3	47.5 (9.05)	<0.01	29.7, 65.2

25(OH)D, 25-hydroxyvitamin D; T, time-point.

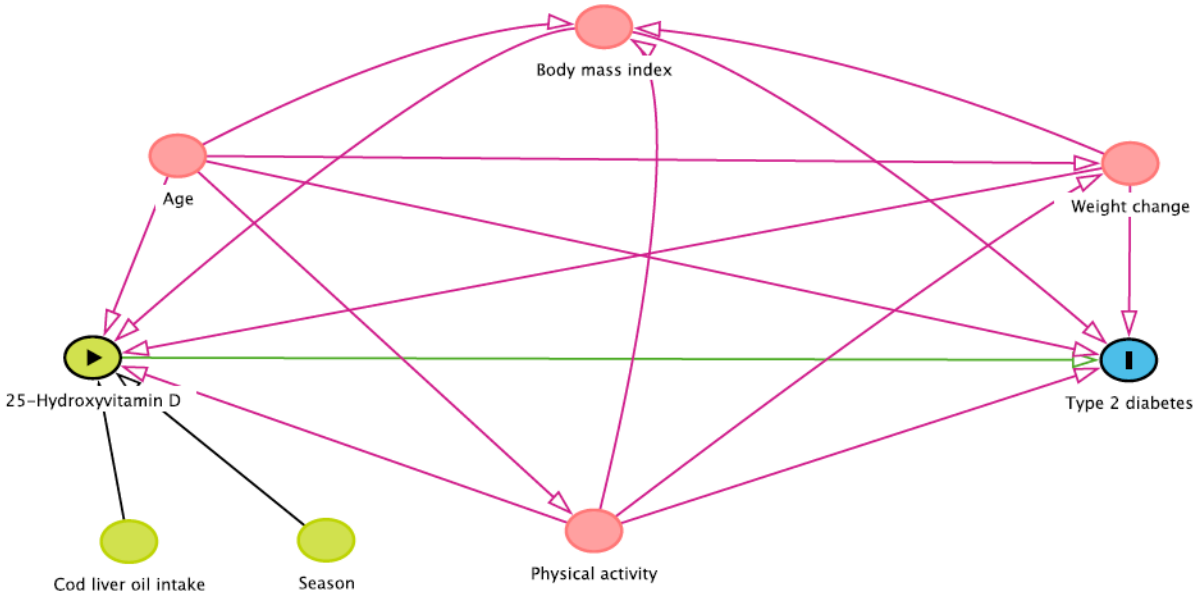

Figure S1. Directed acyclic graph illustrating assumptions about the associations and causal relationships between 25-hydroxyvitamin D and type 2 diabetes. Red circles illustrate confounders, green illustrate exposure, and blue is the outcome.

Paper III

Allaoui G, Rylander C, Fuskevåg OM, Grimnes G, Averina M, Wilsgaard T, Berg V

Longitudinal assessment of classic and 11-oxygenated androgen concentrations and their association with the development of type 2 diabetes – the Tromsø Study.

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Longitudinal assessment of classic and 11-oxygenated androgen concentrations and their association with Type 2 Diabetes Mellitus development – The Tromsø Study

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Abstract

Aim We aimed to investigate changes in pre-diagnostic concentrations of classic and 11-oxygenated androgens in type 2 diabetes (T2DM) cases and healthy controls, associations between androgen concentrations and T2DM, and the potential for androgens to improve the prediction of T2DM when considered in combination with established risk factors.

Methods Androgen concentrations were analysed in serum samples from 116 T2DM cases and 138 controls at three, pre-diagnostic time-points: 1986/87 (T1), 1994/95 (T2), and 2001 (T3). Generalised estimating equations were used to longitudinally examine androgen concentrations, and logistic regression models were used to estimate the odds ratios (OR) of T2DM at each time-point. Logistic regression models were also used to calculate area under the receiver operating characteristics curve (AROC) from models including established risk factors alone (ERF model) and established risk factors plus each androgen, respectively, which were compared to identify improvements in predictive ability.

Results For women, no significant associations were observed between any of the investigated androgens and T2DM after adjusting for confounders. For men, after adjusting for confounders, concentrations of all investigated 11-oxygenated androgens were higher in cases than controls at one or several time-points. We observed associations between T2DM and concentrations of 11-ketoandrostenedione (OR: 1.59) and 11-ketotestosterone (OR: 1.62) at T1; and 11-hydroxyandrostenedione (OR: 2.00), 11-hydroxytestosterone (OR: 1.76), 11-ketoandrostenedione (OR: 1.84), 11-ketotestosterone (OR: 1.78) and testosterone (OR: 0.45) at T3 in men. The addition of these androgens (including 11-hydroxytestosterone at T2) to the ERF model resulted in an improved ability to predict T2DM in men (AROC: 0.79-0.82). We did not observe significant differences in changes in androgen concentrations over time between cases and controls in either sex.

Conclusion Our results demonstrate that testosterone and 11-oxygenated androgens are associated with T2DM in men before diagnosis and may be potential biomarkers in T2DM risk assessment.

Keywords Androgens; Type 2 Diabetes Mellitus; Longitudinal Survey; Preventive; Health Service

Introduction

Disruption of androgen homeostasis has been reported to have a sex-specific association with metabolic dysfunction, insulin resistance, and type 2 diabetes (T2DM) [1, 2]. Androgens are a group of steroid hormones produced in the testes, ovaries, and adrenal glands. They exert their effect by binding to androgen receptors and are integral parts of several processes, including sexual development, body shape, growth, energy expenditure, and modulation of the cardiovascular system (Fig. S1) [3, 4]. It has also recently been reported that 11-oxygenated androgens have greater androgenic activity than previously believed, and several observations point towards a potential role in the development of T2DM [5]. For instance, women with polycystic ovarian syndrome (PCOS) often have higher serum levels of classic and 11-oxygenated androgens compared to healthy women and is associated with insulin resistance and increased risk of T2DM [2, 5-7]. Also, individuals with conditions that cause elevated 11-oxygenated androgens due to 21-hydroxylase deficiency (in both men and women), and those with conditions that cause reduced testosterone levels due to hypogonadism in men, have an increased risk of T2DM [8, 9]. Several cross-sectional and longitudinal studies have reported inconsistent associations between classic androgens and T2DM, where dehydroepiandrosterone sulphate (DHEAS) showed non-significant associations with T2DM in men and women in one study [10], while another observed that lower levels of DHEAS in men were significantly associated with T2DM [11]. Further, in some studies, testosterone concentrations were positively associated with the risk of T2DM in women, but inversely associated in men [12-14], whereas other studies did not find any association [15, 16]. However, there is a lack of studies on 11-oxygenated androgens, and it is in our interest to understand how serum concentrations of androgens change over time and their association with T2DM. Therefore, we aimed to investigate; i) the associations between androgen concentrations and T2DM; ii) pre-diagnostic changes in concentrations of androgens in T2DM cases and controls; and iii) the potential for androgens to improve the prediction of T2DM when considered in combination with established risk factors.

Materials and methods

Study population

The Tromsø Study is a large, ongoing health survey with participants from the Tromsø municipality in Northern Norway. To date, seven surveys of the Tromsø Study (Tromsø1-

Tromsø7) have been conducted between 1974 and 2016. Participants attended various physical examinations, answered questionnaires, and donated blood samples for future research. The Tromsø Study is described in greater detail elsewhere [17].

The present study has a longitudinal, nested case-control design with repeated measurements from participants who attended Tromsø3 (1986/87), Tromsø4 (1994/95), and Tromsø5 (2001), hereafter referred to as time-points 1 (T1), 2 (T2), and 3 (T3), respectively. We included 145 participants who had serum samples available at all time-points and a diagnosis of T2DM after T3 (2001) recorded in a local diabetes registry as cases. We also included 145 controls, who were randomly selected among participants with serum samples available at all time-points and without any T2DM diagnosis at any time-point. Finally, we excluded 29 cases and seven controls with HbA_{1c} levels higher than 48 mmol/mol (6.5%) at any time-point, resulting in a final sample of 116 cases (60 women and 56 men) and 138 controls (75 women and 63 men).

Analysis of androgens

Serum samples, which were collected and frozen at -80ºC at the time of each survey, were thawed and analysed for three classic (A4, DHEAS, testosterone) and four 11-oxygenated androgens (11-hydroxytestosterone, 11OHT; 11-hydroxyandrostenedione, 11OHA4; 11-ketoandrostenedione, 11KA4; 11-ketotestosterone, 11KT). The in-house laboratory method used for androgen analysis is described in detail in the supplementary file (Table S1). Briefly, analyses were performed at the Arctic University of Norway, by liquid-liquid extraction (Tecan Fluent, Männedorf, Switzerland) and liquid chromatography (Waters Acquity™ *I*-class, Waters, Milford, Massachusettes, USA) interfaced with tandem mass spectrometry (Waters Xevo TQ-XS, Waters, Manchester, UK). MassChrom® Steroid panels (Chromsystems Instruments & Chemicals GmbH, München, Germany) and in-house quality controls were included with each run for classic and 11-oxygenated androgens, respectively. All the standards and quality controls were within the acceptance limits of ±15% from target value.

Statistical analysis

Study sample characteristics are reported as means with standard deviations and/or frequencies with percentages, while androgen concentrations are reported as medians with interquartile ranges (IQRs). The distribution of androgen concentrations was assessed visually with histograms and tested for normality with the Shapiro-Wilks test, which indicated that none of the androgens were normally distributed. Differences in the characteristics of cases and controls were compared at each time-point using independent two-sample t-tests or Pearson's $\chi 2$ test, while differences in the androgen concentrations were compared at each time-point using nonparametric Mann-Whitney U-test. All analyses were stratified by sex.

Following a review of the literature [18-23], directed acyclic graphs were constructed to identify potential confounding variables between androgens and T2DM (Fig. S2 and Fig. S3). Potential confounders identified for men were age, body mass index (BMI), and physical activity. The same confounders were identified for women, but also included age at menarche (years), parity (as a proxy for number of pregnancies), menstrual status (no; yes; uncertain/irregular), use of oral/intrauterine contraceptives (no; yes; previously), and use of hormone replacement therapy (no; yes; previously).

Generalised estimating equations, with log-link and gamma distribution to account for non-normality of the response variable, were used to assess androgen concentrations at each time-point and their time-trends. The concentrations were used as continuous dependent variables; T2DM status, the identified confounders, and an indicator variable for time with a two-way interaction term with T2DM, were used as independent variables. Unstructured correlation was used to address within-group correlation for the repeated measurements.

The association between androgen concentrations (per 1-IQR increment) and T2DM was assessed at each time-point by estimating odds ratios (ORs) using logistic regression models. Crude models included the respective androgen concentrations as continuous, independent variable, and T2DM status as the dependent variable. Adjusted models were identical to crude models but adjusted for the identified confounders.

To determine the potential for androgens to improve the prediction of T2DM, we constructed three types of logistic regression models: 1) a model including established risk factors alone (ERF model), 2) one that included established risk factors plus each investigated androgen respectively, and 3) one with a combination of androgens (showed to be significant at any time-point in previous crude logistic regression models), chosen by backwards selection, in addition to established risk factors, with significantly improved model fit (by likelihood-ratio test) as criteria for inclusion of the androgens. ORs for androgen concentrations were evaluated by 1-IQR increments, and the ERF model included: age (continuous), BMI (continuous), physical activity (active: ≥3 h/week of light activity and/or ≥1 h hard exercise/week and sedentary: <3 h/week of activity that provoked transpiration or no activity), elevated blood pressure (systolic blood pressure ≥130, diastolic blood pressure ≥85, and/or use of blood pressure medication, yes/no), and family history of T2DM (siblings and/or parents with T2DM, yes/no) [24]. Models 2 and 3 were then compared to model 1 to identify improvements in predictive ability, assessed by area under the receiver operating characteristics (AROC), and interpreted as follows: an AROC of 0.50 indicates no discrimination, 0.50–0.70 poor discrimination, 0.70–0.80 acceptable discrimination, 0.80–0.90 excellent discrimination, and ≥0.90 outstanding discrimination [25].

Statistical analysis were performed in STATA (v 17.0, StataCorp LLC, 4905 Lakeway Drive, College Station, Texas, USA). Significance level was set at 5% with two-sided *p* values.

Results

Study sample characteristics

Detailed study sample characteristics that were reported in our previous study [26] and additional details about the specific covariates in the present study are included in the supplemental material (Table S2). For both men and women, cases and controls were similar in age, while cases had a significantly higher BMI (3.4-4.9 kg/m^2 higher for women and 2.9-3.3 kg/m^2 for men) at all time-points. Cases and controls had similar physical activity levels at all time-points, except at T2, when cases were less active than controls. For women, a higher percentage of cases than controls had elevated blood pressure and a family history of T2DM, while there were no significant differences in men. There were no significant differences in age at menarche, parity, menstrual status, or use of oral/intrauterine contraceptives in women.

Androgen concentrations

For women, concentrations of 11OHT at T2 and T3 were significantly higher in cases than in controls; whereas for men, concentrations of 11OHT, 11OHA4, 11KA4, and 11KT were higher, and testosterone was lower at all time-points in cases than in controls (Fig. 1 and Table S3). After adjusting for confounders, no significant differences remained between women cases and controls; whereas for men, the differences between cases and controls remained significant for 11OHT (T2, T3), 11OHA4 (T3), 11KA4 (T1, T3), and 11KT (T1-T3) (Table S4). No significant differences between cases and controls were found for A4 and DHEAS.

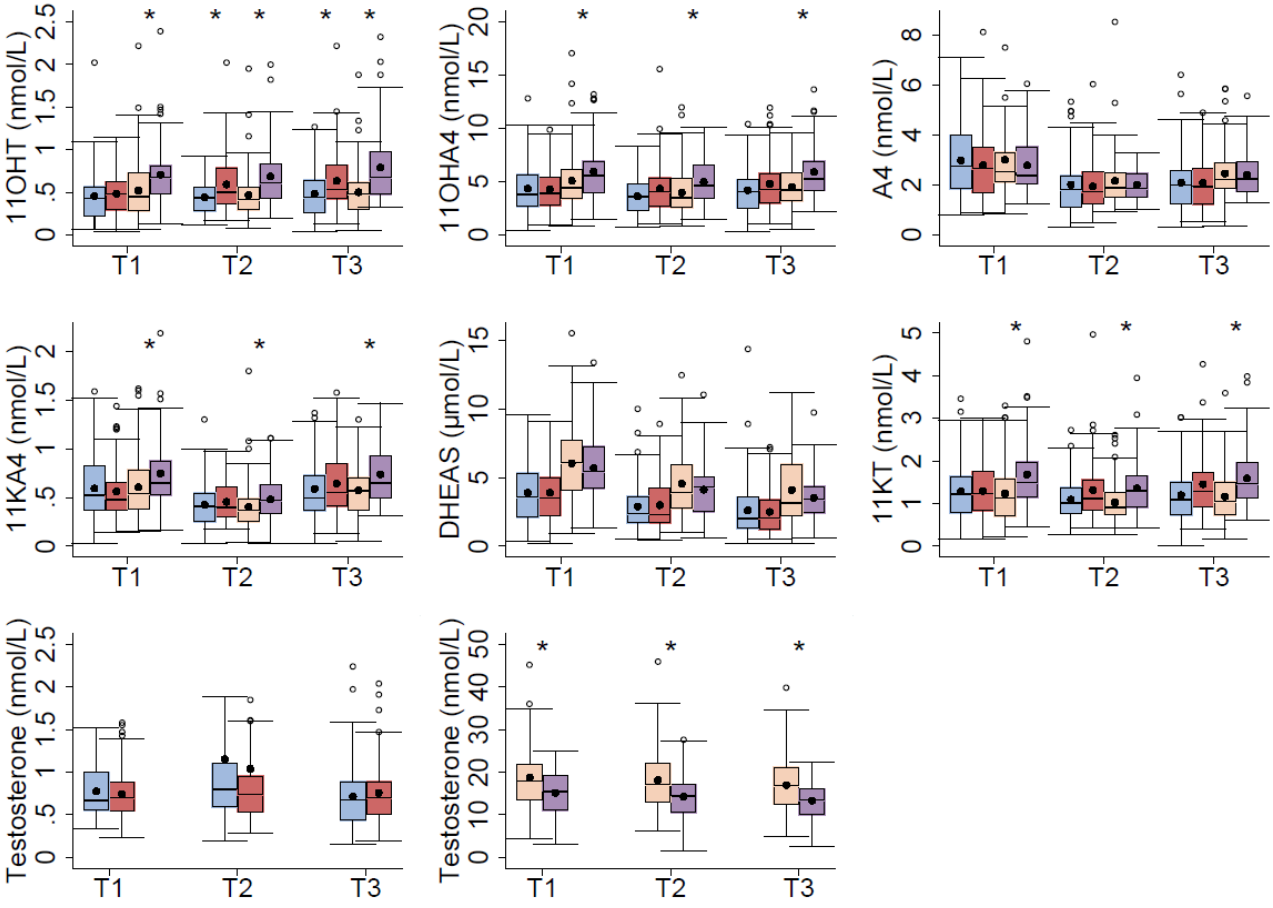


Fig. 1. Androgen concentrations across all time-points in women (cases in red and controls in blue) and men (cases in purple and controls in orange). The Tromsø Study 1986–2001. Sample numbers at each time point: women: 60 cases and 75 controls; men: 56 cases and 63 controls. 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrostenedione; A4, androstenedione; DHEAS,

dehydroepiandrosterone. T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

* $p<0.05$

Longitudinal changes in androgen concentrations

For both men and women, no significant differences in changes in androgen concentrations over time were observed in cases compared to controls after adjusting for confounders (Fig. 2 and 3, Table S4).

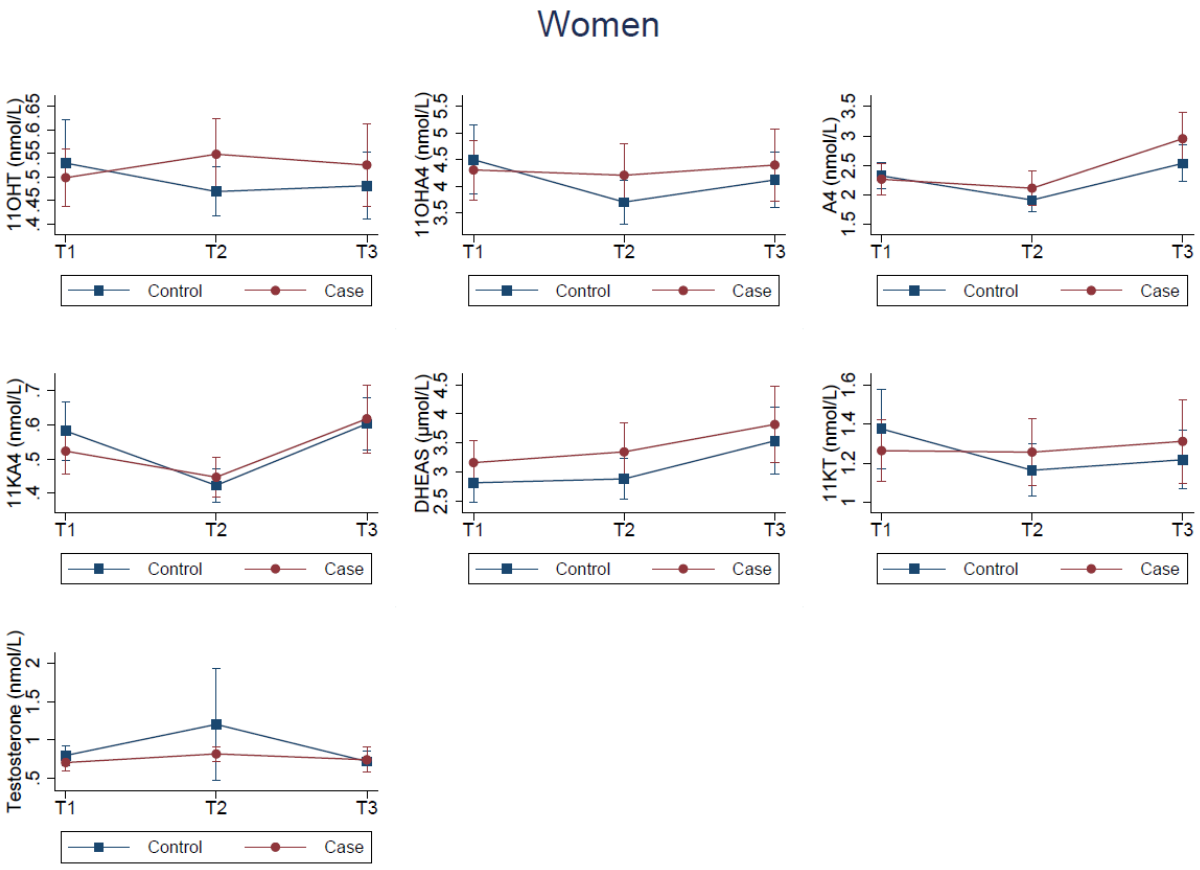


Fig. 2. Estimated mean androgen concentrations across T1, T2, and T3 in women (60 cases and 75 controls at each time-point). The Tromsø Study 1986–2001. The estimated means in cases (red, circle) and controls (blue, square) are adjusted for age, body mass index, physical activity, age at menarche, parity, menstrual status, use of oral/intrauterine contraceptives, and use of hormone replacement therapy. T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

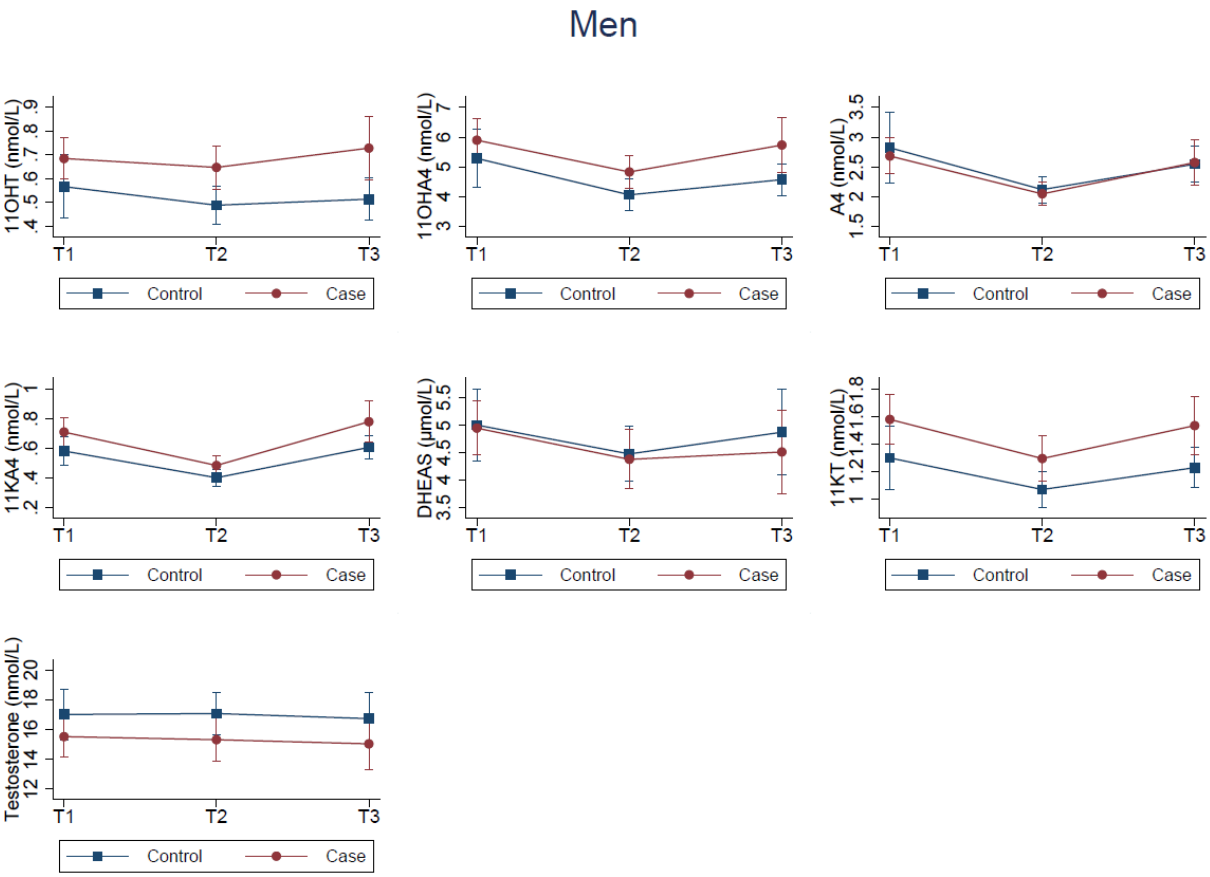
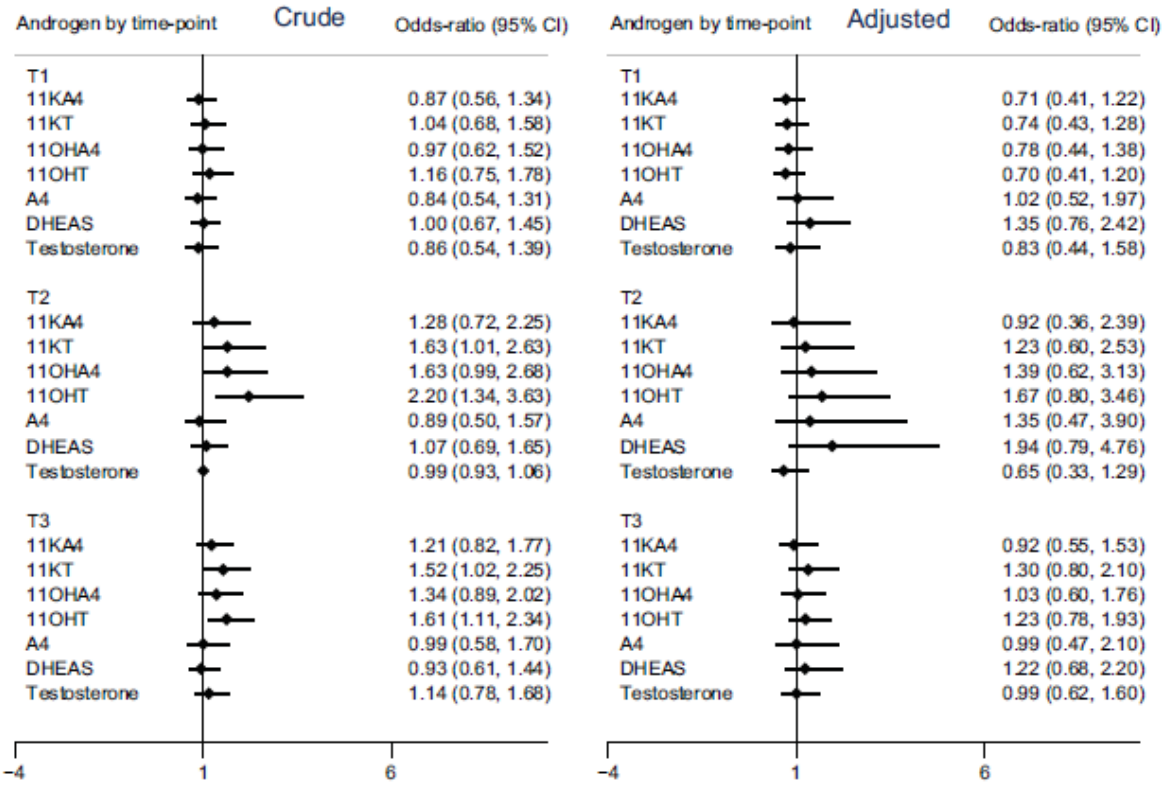


Fig. 3. Estimated mean androgen concentrations across T1, T2, and T3 in men (56 cases and 63 controls at each time-point). The Tromsø Study 1986–2001. The estimated means for cases (red, circle) and controls (blue, square) are adjusted for age, body mass index, and physical activity. T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

Associations between androgen concentrations and T2DM

No significant associations were found between androgen concentrations and T2DM in the adjusted models for women (Fig. 4). For men, each 1-IQR increment in 11KA4 (T1, T3), 11KT (T1, T3), 11OHT (T3), and 11OHA4 (T3) significantly increased the OR of T2DM, while each 1-IQR increment in testosterone (T3) decreased these OR (Fig. 4 and Table S5).

Women


Androgen by time-point
Crude
Odds-ratio (95% CI)
T1
11KA4 0.87 (0.56, 1.34)
11KT 1.04 (0.68, 1.58)
11OHA4 0.97 (0.62, 1.52)
11OHT 1.16 (0.75, 1.78)
A4 0.84 (0.54, 1.31)
DHEAS 1.00 (0.67, 1.45)
Testosterone 0.86 (0.54, 1.39)
T2
11KA4 1.28 (0.72, 2.25)
11KT 1.63 (1.01, 2.63)
11OHA4 1.63 (0.99, 2.68)
11OHT 2.20 (1.34, 3.63)
A4 0.89 (0.50, 1.57)
DHEAS 1.07 (0.69, 1.65)
Testosterone 0.99 (0.93, 1.06)
T3
11KA4 1.21 (0.82, 1.77)
11KT 1.52 (1.02, 2.25)
11OHA4 1.34 (0.89, 2.02)
11OHT 1.61 (1.11, 2.34)
A4 0.99 (0.58, 1.70)
DHEAS 0.93 (0.61, 1.44)
Testosterone 1.14 (0.78, 1.68)
−4
1
6
Androgen by time-point
Adjusted
Odds-ratio (95% CI)
T1
11KA4 0.71 (0.41, 1.22)
11KT 0.74 (0.43, 1.28)
11OHA4 0.78 (0.44, 1.38)
11OHT 0.70 (0.41, 1.20)
A4 1.02 (0.52, 1.97)
DHEAS 1.35 (0.76, 2.42)
Testosterone 0.83 (0.44, 1.58)
T2
11KA4 0.92 (0.36, 2.39)
11KT 1.23 (0.60, 2.53)
11OHA4 1.39 (0.62, 3.13)
11OHT 1.67 (0.80, 3.46)
A4 1.35 (0.47, 3.90)
DHEAS 1.94 (0.79, 4.76)
Testosterone 0.65 (0.33, 1.29)
T3
11KA4 0.92 (0.55, 1.53)
11KT 1.30 (0.80, 2.10)
11OHA4 1.03 (0.60, 1.76)
11OHT 1.23 (0.78, 1.93)
A4 0.99 (0.47, 2.10)
DHEAS 1.22 (0.68, 2.20)
Testosterone 0.99 (0.62, 1.60)
−4
1
6

Men

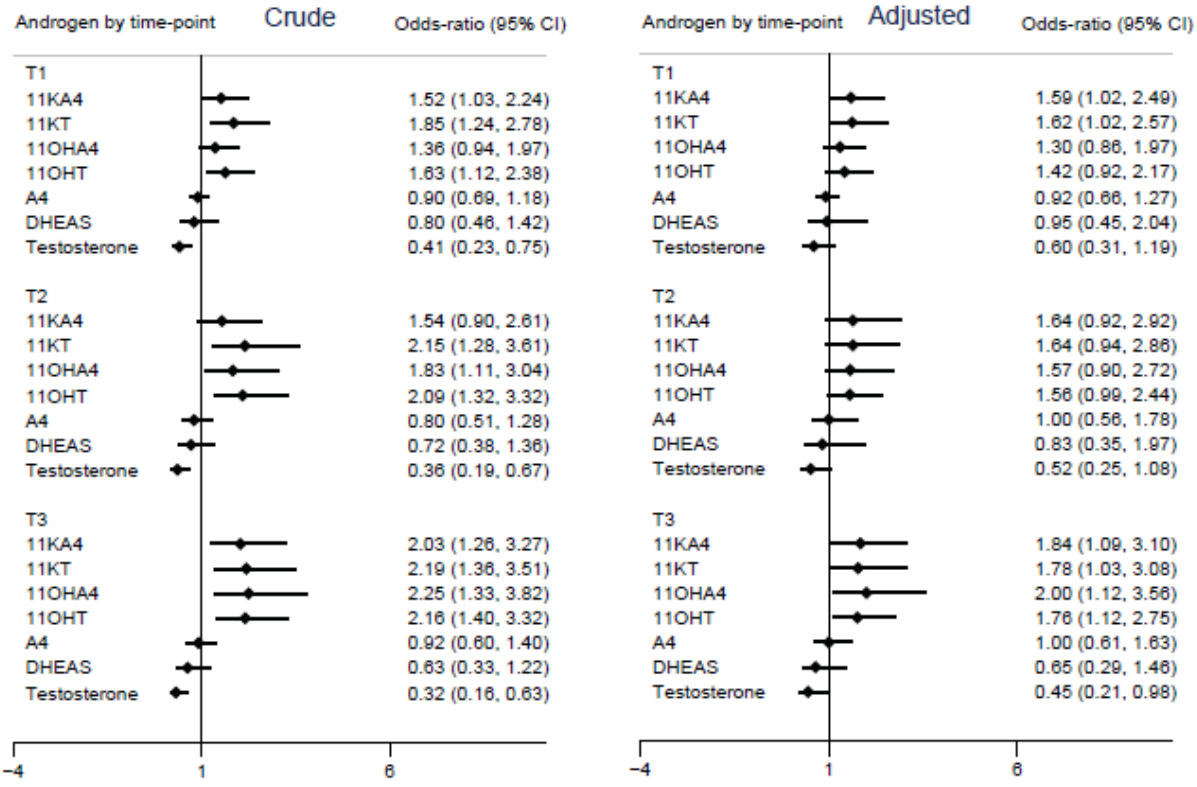

Androgen by time-point
Crude
Odds-ratio (95% CI)
T1
11KA4 1.52 (1.03, 2.24)
11KT 1.85 (1.24, 2.78)
11OHA4 1.36 (0.94, 1.97)
11OHT 1.63 (1.12, 2.38)
A4 0.90 (0.69, 1.18)
DHEAS 0.80 (0.46, 1.42)
Testosterone 0.41 (0.23, 0.75)
T2
11KA4 1.54 (0.90, 2.61)
11KT 2.15 (1.28, 3.61)
11OHA4 1.83 (1.11, 3.04)
11OHT 2.09 (1.32, 3.32)
A4 0.80 (0.51, 1.28)
DHEAS 0.72 (0.38, 1.36)
Testosterone 0.36 (0.19, 0.67)
T3
11KA4 2.03 (1.26, 3.27)
11KT 2.19 (1.36, 3.51)
11OHA4 2.25 (1.33, 3.82)
11OHT 2.16 (1.40, 3.32)
A4 0.92 (0.60, 1.40)
DHEAS 0.63 (0.33, 1.22)
Testosterone 0.32 (0.16, 0.63)
−4
1
6
Androgen by time-point
Adjusted
Odds-ratio (95% CI)
T1
11KA4 1.59 (1.02, 2.49)
11KT 1.62 (1.02, 2.57)
11OHA4 1.30 (0.86, 1.97)
11OHT 1.42 (0.92, 2.17)
A4 0.92 (0.66, 1.27)
DHEAS 0.95 (0.45, 2.04)
Testosterone 0.60 (0.31, 1.19)
T2
11KA4 1.64 (0.92, 2.92)
11KT 1.64 (0.94, 2.86)
11OHA4 1.57 (0.90, 2.72)
11OHT 1.56 (0.99, 2.44)
A4 1.00 (0.56, 1.78)
DHEAS 0.83 (0.35, 1.97)
Testosterone 0.52 (0.25, 1.08)
T3
11KA4 1.84 (1.09, 3.10)
11KT 1.78 (1.03, 3.08)
11OHA4 2.00 (1.12, 3.56)
11OHT 1.76 (1.12, 2.75)
A4 1.00 (0.61, 1.63)
DHEAS 0.65 (0.29, 1.46)
Testosterone 0.45 (0.21, 0.98)
−4
1
6

Fig. 4. Forest plots illustrating crude and adjusted OR of type 2 diabetes for each androgen by time-point and sex. The Tromsø Study 1986–2001. Adjusted models included age, body mass index, and physical activity for both sexes. Models for women were further adjusted for age at menarche, parity, menstrual status, and use of oral/intrauterine contraceptives.
OR for all androgens are estimated per 1-IQR increment. Sample numbers at each time point: women: 60 cases and 75 controls; men: 56 cases and 63 controls. 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrostenedione; A4, androstenedione; CI: confidence interval; DHEAS, dehydroepiandrosterone; IQR, interquartile range; T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

Potential of androgens to improve the prediction of T2DM

For men, the addition of 11KA4 or 11KT to the ERF model significantly improved the discrimination between cases and controls at T1 (79% versus 78% for the ERF model) (Table 1). Similar results were observed at T2 for the addition of 11OHT (80% discrimination versus 76% for the ERF model). At T3, the addition of 11OHT, 11OHA4, 11KA4, 11KT, or testosterone to the ERF model improved the discrimination between cases and controls (79-82% versus 78%).

Finally, when a combination of 11OHT and testosterone at T3 was added to the ERF model, a significant improvement over the models already presented was observed, showing 85% discrimination. At all other time-points, for both men and women, no androgen combinations improved discrimination or model fit more than those already presented.

Table 1. Ability of models including established risk factors alone, and established risk factors in addition to androgens, to predict T2DM across pre-diagnostic time-points. The Tromsø Study 1986-2001.

		T1	**T2**	**T3**
	Sex	**AROC**	**AROC**	**AROC**
Established risk factors[a]	Women	0.77	0.81	0.80
	Men	0.78	0.76	0.78
11-hydroxytestosterone[b] (nmol/l)	Women	0.77	0.82	0.80
	Men	0.79	0.80*	0.82*

11-hydroxyandrostenedione[b] (nmol/l)	Women	--	--	--
	Men	0.78	0.79	0.81*
11-ketoandrostenedione[b] (nmol/l)	Women	--	--	--
	Men	0.79*	0.78	0.81*
11-ketotestosterone[b] (nmol/l)	Women	0.77	0.81	0.80
	Men	0.79*	0.78	0.81*
Testosterone[b] (nmol/l)	Women	--	--	--
	Men	0.78	0.78	0.79*
Combined model[c]	Women	--	--	--
	Men	--	--	0.85*

AROC, Area under the receiver operating characteristics; T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

[a]Established risk factors include age (continuous), body mass index (continuous), physical activity (active: ≥3 h/week of light activity and/or ≥1 h hard exercise/week or sedentary: <3 h/week of activity that provoked transpiration or no activity), elevated blood pressure (systolic blood pressure ≥130, diastolic blood pressure ≥85, and/or if the subject was taking blood pressure medication, yes/no), and family history of type 2 diabetes (siblings and/or parents with type 2 diabetes, yes/no).

[b]Androgen included with the established risk factors.

[c]Combined model for men at T3: established risk factors, 11OHT, and testosterone.

*Significant improvement in model fit by likelihood-ratio test between confounders and respective androgen plus confounders.

Discussion

To the best of our knowledge, this is the first study to address the relationship between pre-diagnostic measures of classic and 11-oxygenated androgens and T2DM with repeated measurements over a 15-years period. In men, after adjusting for confounders, cases had higher concentrations of 11-oxygentated androgens compared to controls throughout the study period and were positively associated with T2DM at T3 before its diagnosis in cases, whereas testosterone concentration was negatively associated with T2DM at T3. For women, after adjusting for confounders, there were no significant differences in androgen concentrations between cases and controls. In men, the addition of 11KA4, 11KT, 11OHT, 11OHA4, and testosterone, respectively, to the ERF model significantly improved discrimination between cases and controls, with acceptable to excellent discrimination (AROC 0.79-0.82) compared to acceptable discrimination (AROC 0.76-0.78) for the ERF model across all time-points. The strongest gain in discrimination was achieved by adding both 11OHT and testosterone to the ERF model at T3, with excellent discrimination (AROC 0.85) between cases and controls.

We observed that, in men, cases had significantly lower testosterone levels at all time-points compared to controls, and at T3 was significantly associated with T2DM after adjusting for confounders. This agrees with previous studies and meta-analysis, which found that decreased testosterone levels were associated with T2DM [12, 27]. No association was found between testosterone and T2DM for women, which also agrees with a previous meta-analysis [28]. In contrast to our results, some have observed that higher testosterone levels in women are associated with T2DM [12, 13]. Conversely, a study by O'Reilly et al. [12] with a median follow-up of 3.2-3.3 years found that lower levels of testosterone significantly increased the risk of T2DM for men (n: 70541, mean age: 51.6 years) and that higher levels of testosterone increased the risk of T2DM for women (n: 81889, mean age: 33.2). Further, for women, they found that testosterone levels above 1.5 nmol/l increased the risk significantly. In the present study, the highest median testosterone concentration was 0.80 nmol/l, and few women had testosterone concentrations above 1.5 nmol/l, which may explain the discrepancy between these two studies. With 10 years of follow-up, Ding et al. [13] found a significant increase in the risk of T2DM in women with testosterone levels above 1.15 nmol/l (359 T2DM cases vs 359 controls, mean age: 60.3 years), which further indicates that testosterone levels higher than those seen in the present study are associated with T2DM.

Studies that have investigated the associations between 11-oxygenated androgens and T2DM in prospective cases are non-existent. In a study by Davio et al. [29], the aim was to compare 11-oxygenated androgen concentrations across adulthood in men and women in a general population, and they observed no associations with hyperglycaemia or T2DM in men and women. These findings agree with the non-significant associations we found in women, but not with our results in men. However, Davio et al. had a cross-sectional design that included prevalent T2DM cases (68 cases, 455 controls), whereas we studied T2DM cases before diagnosis. Some studies have examined the correlation between 11-oxygenated androgens and insulin, insulin resistance, and/or insulin sensitivity, however none of them included men. O'Reilly et al. [30] found that concentrations of 11OHA4 and 11KA4 were positively correlated with insulin and insulin resistance among healthy women and those with PCOS, and Walzer et al. [31] observed that, in women with lipodystrophy, 11OHA4, 11KA4, and 11KT, but not 11OHT, were associated with increased insulin signalling due to hyperinsulinemia as a response to insulin resistance. In contrast, Tosi et al. [6] found a positive correlation between insulin sensitivity and 11OHT and 11KT among women with PCOS. As disrupted insulin homeostasis is the major metabolic abnormality in T2DM, these observed correlations could be assumed to exist between 11-oxygenated androgens and T2DM.

In men, the addition of 11-oxygenated androgens and testosterone to the ERF model improved the prediction of T2DM (as early as 15 years before diagnosis for 11KA4 and 11KT). This suggests that elevated 11-oxygenated androgen concentrations and decreased testosterone concentrations might serve as biomarkers to identify individuals at high risk of T2DM when considered along with established risk factors. A study by Atlantis et al. [32] (n=1655, median follow-up 4.95 years) compared a risk model consisting of combined variables from several risk models, with a risk model that additionally included testosterone and observed an increase in AROC from 0.82 to 0.83. They concluded that the discrimination was not significantly improved, but as testosterone remained significant, they presumed that its addition is valuable for identifying high-risk individuals.

In the present study, there were no significant differences in changes in androgen concentrations over time between cases and controls. This means that cases in men had higher

concentrations compared to controls at T1, but the differences remained constant over the 15-year study period. These similar changes might be explained by the physiological properties of hormones. Indeed between-individual variations are generally greater than within-individual variations, and hormone homeostasis is tightly regulated within each individual by feedback mechanisms, thus a large difference in changes over time between cases and controls would not be expected [4]. The similar changes in androgens over time in cases and controls could indicate that androgen homeostasis was not disturbed during the study period. We do not know if the androgen concentrations were similar in cases and controls at an earlier time-point, and if so, when the difference in concentrations developed or what caused it. Hence, we cannot conclude whether the difference in androgen concentrations affected T2DM progression, or if it was a consequence of processes related to disease development.

Among men, cases had higher concentrations of 11-oxygenated androgens compared to controls. The mechanisms behind this increased concentration may be explained by an increase in A4 conversion to testosterone by the androgen-activating enzyme aldo-ketoreductase type 1 C3 (AKR1C3), which in turn could increase the downstream production of 11-oxygenated androgens by, for example, cytochrome P450 family 11 subfamily B member 1 (CYP11B1; Fig. S1). T2DM cases often have increased adipose tissue as well as increased insulin levels (e.g., due to insulin resistance). As AKR1C3 expression and activity has been observed to be increased in subcutaneous adipose tissue and by increased insulin levels, it can be hypothesised that AKR1C3 expression is increased in individuals with T2DM [33-35]. Another explanation for the increase in 11-oxygenated androgens in the cases could be that steroidogenesis is upregulated by insulin in general [36]. For example, the enzyme CYP11B1 (expressed mainly in the adrenal glands), which converts A4 and testosterone to 11OHA4 and 11OHT, respectively, has been reported to be upregulated by insulin [36, 37].

The concentrations of androgens reported in this study are comparable to concentrations reported in healthy subjects in several other studies [4, 6, 29-31, 38-41]. Even though we did not find any clear associations between androgens and T2DM in women in our study, associations have been observed in studies of women with PCOS, who have an excess of androgens, insulin sensitivity/resistance, and increased risk of developing T2DM [6, 29, 30, 38, 39, 42-44]. The study by O'Reilly et al. [30], who observed significant relationships

between insulin resistance and 11-oxygenated androgens, also observed that women with PCOS had concentrations between 1.34-5.0 times higher than controls. Androgen concentrations among women with T2DM in the present study were 0.9-1.2 times higher than in controls, which implies that androgen concentrations in women must be higher to detect significant associations and to be considered risk factors.

A major strength of this study is its nested case-control study design, with three repeated measurements available up to 15 years before T2DM diagnosis, and the wide array of data collection. T2DM diagnosis was determined by local diabetes registries and confirmed by medical records and HbA_{1c} results. Androgen measurements were based on analyses of serum samples by LC-MS/MS. Limitations include that external controls were only available for A4, DHEAS, and testosterone, but we did have in-house spiked controls for all androgens. We were not able to control for the time of blood sample collection; however, all participants followed the same enrolment procedures, thus cases and controls were treated equally, resulting in similar blood sampling times. Stratifying by sex and time-point in the logistic regression models might have hampered the precision of our estimates and thus affected the interpretation of the results' significance. The results are based on a northern Norwegian population and given the lack of studies and clinically relevant reference ranges for 11-oxygenated androgens, future studies are needed to re-evaluate the validity of our results.

Conclusions

For men, prospective T2DM cases had consistently higher concentrations of 11-oxygenated androgens, and lower testosterone concentrations compared to controls. Further, several androgens improved the discrimination of cases and controls in prediction models, indicating that androgens may be potential biomarkers in T2DM risk assessment. Still, we cannot conclude if androgens affect T2DM progression, or whether concentrations are affected by other factors related to disease development.

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Data availability The dataset used in this study was procured from the Tromsø Study and is not publicly available. Access may be obtained by application to the Tromsø Study (https://uit.no/research/tromsostudy).

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Author contribution VB and CR conceived and designed the study. GA and VB contributed to acquiring the data. GA and OMF analysed the data. GA and VB contributed to the interpretation of the data and wrote the manuscript. CR, TW, and GG contributed to the interpretation of data and results, and to the revision of the manuscript. OMF and MA contributed to the laboratory and clinical aspects while drafting and revising the manuscript. OMF also contributed to a more detailed description of the laboratory methods.

Declaration

Conflict of interest The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

Human and Animal Rights disclosure Informed consent was received at each survey from all the participants. The Regional Ethics Committee, REK, Nord approved the study protocol (REK reference: 2015/1780/ REK Nord).

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Supplemental material

Longitudinal assessment of classic and 11-oxygenated androgen concentrations and their association with Type 2 Diabetes Mellitus development – The Tromsø Study

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Method description for liquid chromatography – tandem mass spectrometry setup (LC-MS/MS)

Chemicals

LC-grade tert-butyl methyl ether (TBME) and LC-MS/MS-grade methanol was purchased from Merck KGaA (Dramstadt, Germany), zinc sulphate heptahydrate ($ZnSO_4*7H_2O$) from Acros Organics, LC-MS/MS-grade formic acid from Thermo Scientific, and Milli-Q grade water was produced by a Millipore system.

The analytes 17a-hydroxyprogesterone (**1**), testosterone (**2**), progesterone (**3**), 21-deoxycortisol (**4**), 11-deoxycortisol (**5**), corticosterone (**6**), and cortisol (**7**) were purchased from Cerilliant Corporation (Round Rock, Texas, USA); androstenedione (**8**) from LGC Germany; 4-androsten-11B-17B-diol-3one (11-hydroxytestosterone) (**9**), 4-androsten-11B-OL3-17-dione (11-hydroxyandrostenedione) (**10**), 4-androsten-3-11-17-trione (11-ketoandrostenedione (**11**), 4-androsten-17B-OL3-11B-dione (11-ketotestosterone) (**12**), from Steraloids Inc. (Newport, Rhode Island, USA); and dehydroepiandrosterone sulphate (**13**) from Steraloids Inc. (Newport, Rhode Island, USA).

The isotope labelled analytes 17a-hydroxyprogesterone-d8 (**1***), testosterone-d3 (**2***), progesterone-d9 (**3***), 21-deoxycortisol-d8 (**4***), 11-deoxycortisol-d5 (**5***), corticosterone-d8 (**6***), and dehydroepiandrosterone sulphate-d5 (**13***) were purchased from Cerilliant Corporation (Round Rock, Texas, USA); cortisol-d4 (**7***) from IsoSciences (Ambler, Pennsylvania, USA); androstenedione-d3 (**8***) from TRC Canada (Toronto, Ontario, Canada); 4-androsten-11B-17B-diol-3one-d4 (11-hydroxytestosterone-d4) (**9***), 4-androsten-11B-OL3-17-dione-d4 (11-hydroxyandrostenedione-d4) (**10***), and 4-androsten-3-11-17-trione-d10 (11-ketoandrostenedione-d10) (**11***) from Cambridge Isotope Laboratories (Andover, Massachusetts, USA); and 4-androsten-17B-OL3-11B-dione-d3 (11-ketotestosterone-d3 (**12***) from Cayman Chemical Group (Ann Arbor, Michigan, USA).

For quality control (QC) of analytes **1-8** and **13**, the CE-IVD MassChrom® Steroids panel 1 and 2 with three levels each were purchased from Chromsystems Instruments & Chemicals (München, Germany).

Sample preparation

A seven-point calibration curve ranging from 0.13 to 130 nM for analytes **1, 2, 3, 5, 6,** and **8**; from 0.013 to 13 nM for analyte **4**; from 2.08 to 2080 nM for analyte **7;** from 0.025 to 25 nM for analytes **9, 10, 11,** and **12**; and from 26 to 26000 nM for analyte **13** was prepared in methanol:water (1:1). The isotope labelled analytes were used as internal standards (IS) and were mixed in a concentration of 30, 3, and 1000 nM, respectively, in ultrapure H_2O.

Extraction was performed on a Tecan Fluent 780 liquid handler. 70 µl of sample, calibration standard, and QC samples were transferred to a 96-well plate (Sarstedt) whereafter 60 µl IS-mix was added and 110 µl 0.1 M $ZnSO_4$:methanol (1:1) for protein precipitation. After shaking at 1500 RPM for 2 minutes, 500 µl TBME was added, and the samples were shaken at 1450 RPM for 3 minutes for liquid-liquid extraction of steroids. The plates were centrifuged for 4 minutes at 1600 RPM (Hettich Rotina 320R) and the upper organic phase was transferred to a 1 ml, 96-well sample collection plate (Waters). The solvent was evaporated under a stream of nitrogen while kept on 40°C. Finally, the samples were reconstituted in 60 µl of 70% methanol with 0.1% formic acid.

LC-MS/MS analysis

Separation was achieved with a Cortecs T3, 120Å, 1.6 µm 2.1 x 100 mm (Waters) column, maintained at 50°C with a flow rate of 0.3 mL/min. A linear gradient system composed of 0.1% formic acid and 5

mM ammonium acetate in water, and 0.1% formic acid and 5 mM ammonium acetate in methanol:acetonitrile (1:1) was used, starting from 40% (v/v) methanol, and increasing to 70% in 8 min, maintaining at 95% for 0.5 min before returning to the starting conditions. The autosampler temperature was 6°C and the sample injection volume was 4 µl. The injector was a flow-through-needle (FTN), the needle wash and purge solvent were 90% methanol, and the needle was washed for 6 seconds after injection. Mass spectral data was acquired on a Waters Xevo TQ-XS mass spectrometer (Waters, Manchester, UK) in ESI positive and negative mode with the following conditions: capillary voltage: 1 kV, desolvation gas temperature: 550°C, source temperature: 150°C, desolvation gas flow: 1000 L/hr, cone gas flow: 150 l/hr, nebuliser pressure: 7 Bar. Table S1 shows the retention time (RT), multiple reaction monitoring (MRM) transitions, cone voltage and collision energy used for the different analytes. Data were acquired and analysed using MassLynx version 4.2.

Table S1. Retention time (RT), multiple reaction monitoring (MRM) transitions, cone voltage, and collision energy used for the different analytes.

nr	**Analyte**	**RT (min)**	**Quantifier MRM transitions (m/z)**	**Qualifier MRM transitions (m/z)**	**Cone (V)**	**Collision (eV)**
1	17a-OH-progesterone	6.74	331.1>97	331.1>109	40	23/23
1*	17a-OH-progesterone-d8	6.66	339.1>100			23
2	Testosterone	6.31	289.1>97	289.1>109	40	24/24
2*	Testosterone-d3	6.26	292.1>97	292.1>109		24/24
3	Progesterone	8.37	315.1>109	315.1>97	40	23/23
3*	Progesterone-d9	8.27	324.1>113	324.1>100		23/23
4	21-Deoxycortisol	4.77	347.1>121	347.1>311	20	21/21
4*	21-Deoxycortisol-d8	4.69	355.4>113	355.4>319		18/21
5	11-Deoxycortisol	4.99	347.1>109	347.1>97	40	23/24
5*	11-Deoxycortisol-d5	4.93	352.1>113	352.1>100		24/24
6	Corticosterone	4.77	347.2>329	347.2>121	20	18/20
6*	Corticosterone-d8	4.69	355.2>337	355.2>125		21/21
7	Cortisol	3.63	363.1>121	363.1>327	40	25/14
7*	Cortisol-d4	3.60	367.1>121	367.1>331		25/14
8	Androstenedione	5.78	287.1>109	287.1>97	40	23/23
8*	Androstenedione-d3	5.73	290.1>109	290.1>100		23/23
9	4-Androsten-11B-17B-diol-3one	4.60	305.2>121	305.2>287	40	17/17
9*	4-Androsten-11B-17B-diol-3one-d4	4.56	309.2>121	309.2>291		17/17
10	4-Androsten-11B-OL3-17-dione	4.22	303.2>267	303.2>285	40	18/18
10*	4-Androsten-11B-OL3-17-dione-d4	4.18	307.2>270	307.2>289		21/21
11	4-Androsten-3-11-17-trione	3.46	301.2>257	301.2>121	40	23/23
11*	4-Androsten-3-11-17-trione-d10	3.41	311.2>125	311.2>265		23/23
12	4-Androsten-17B-OL3-11B-dione	3.89	303.2>121	303.2>259	40	23/23
12*	4-Androsten-17B-OL3-11B-dione-d3	3.86	306.2>121	306.2>262		23/23
13	Dehydroepiandrosterone sulphate	2.10	367.1>97			
13*	Dehydroepiandrosterone sulphate-d5		372.1>98			

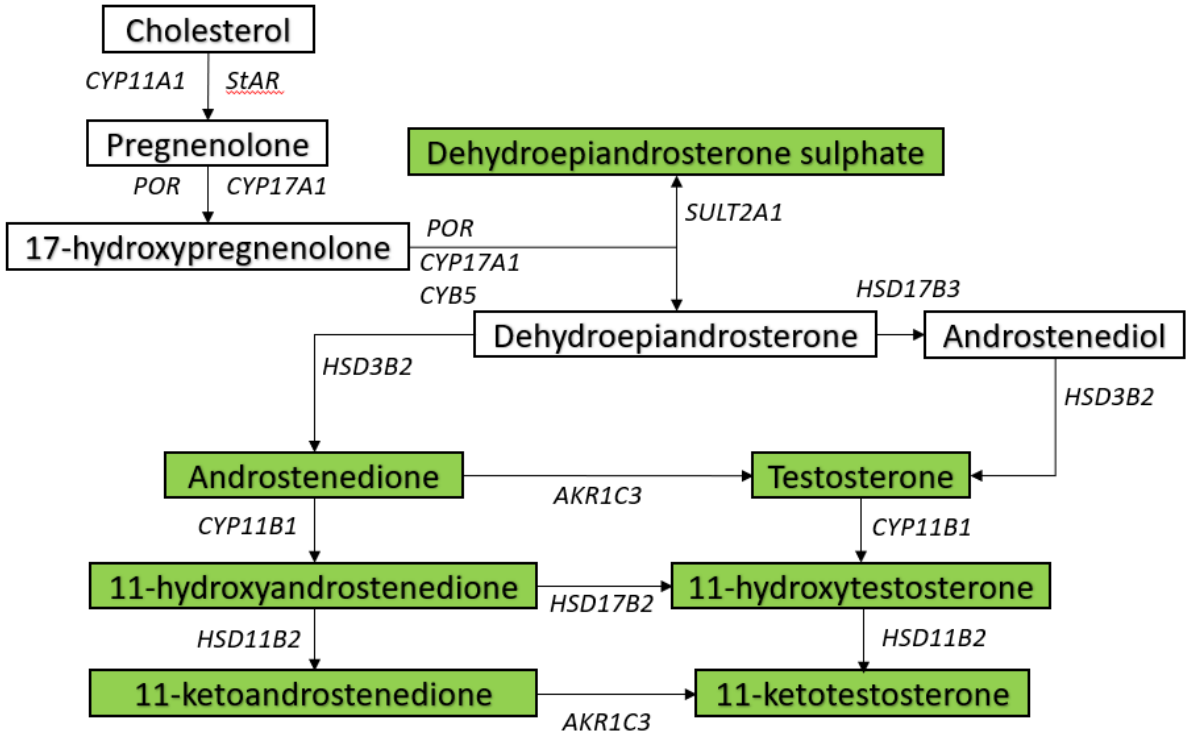

Fig. S1. Pathway schematic of androgen biosynthesis with corresponding genes for the key enzymes in the steroidogenic pathway. AKR1C3, Aldo-keto reductase family 1 member C3; CYB5, cytochrome b_5; CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP11B1, cytochrome P450 family 11 subfamily B member 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; HSD3B2, 3 beta-hydroxysteroid dehydrogenase type 2; HSD11B2, 11 beta-hydroxysteroid dehydrogenase type 2 HSD17B2, 17 beta-hydroxysteroid dehydrogenase type 2; HSD17B3, 17 beta-hydroxysteroid dehydrogenase type 3; POR, cytochrome P450 oxidoreductase; StAR, steroidogenic acute regulatory protein; SULT2A1, sulfotransferase family 2A member 1.

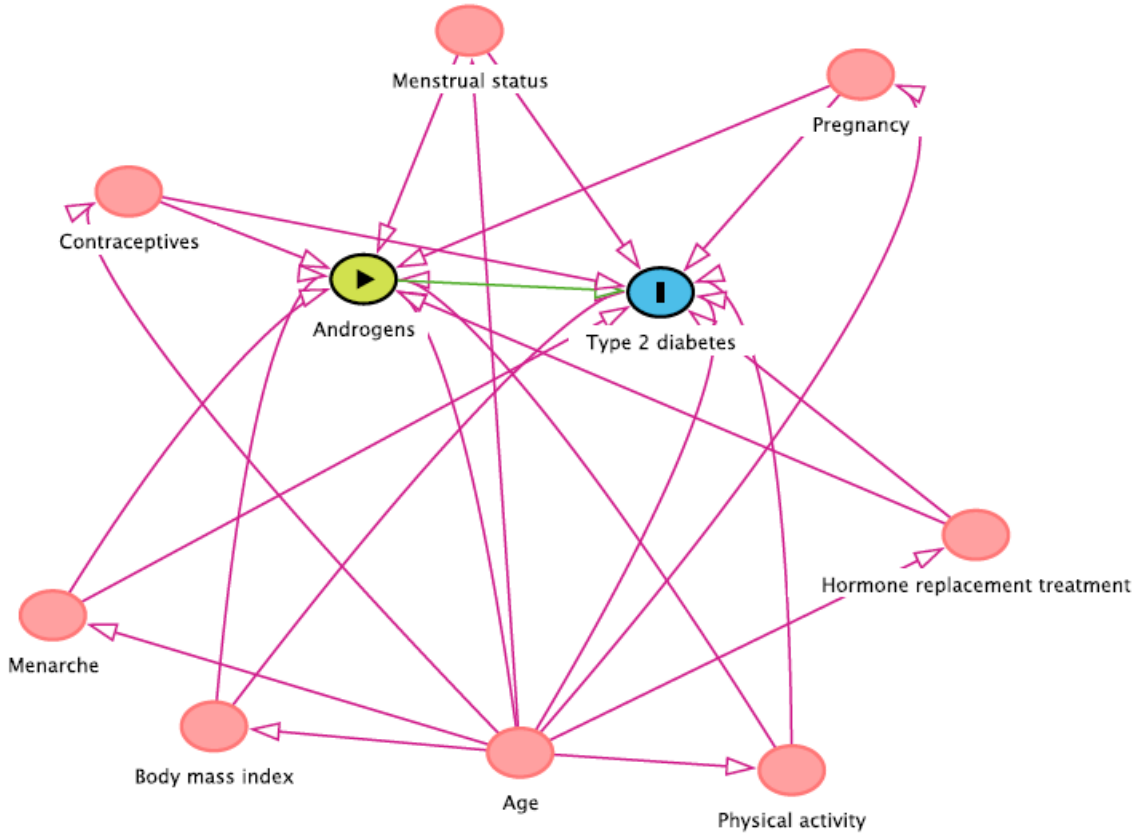

Fig. S2. Directed acyclic graph of assumptions of associations between androgens and type 2 diabetes for women, illustrating potential confounders. Red circles illustrate confounders, the green circle illustrates the exposure, and the blue circle is the outcome.

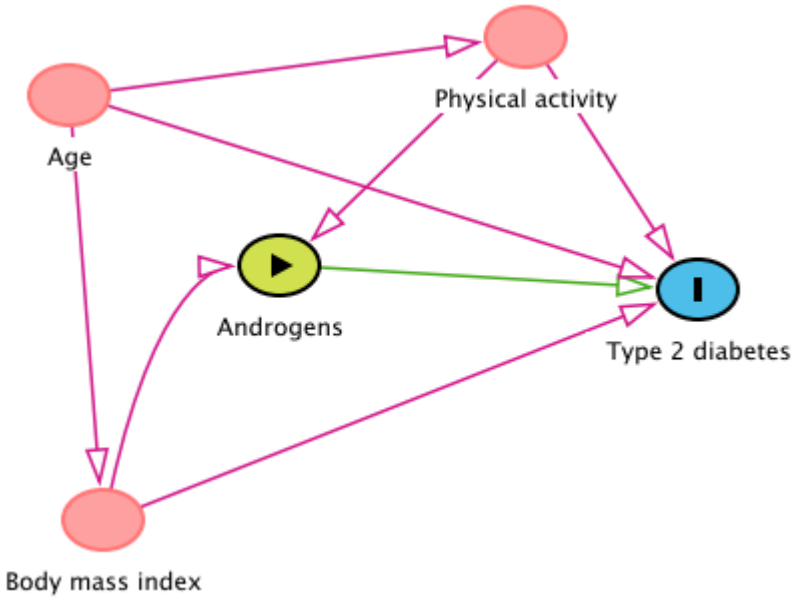

Fig. S3. Directed acyclic graph of assumptions of associations between androgens and type 2 diabetes for men, illustrating potential confounders. Red circles illustrate confounders, the green circle illustrates the exposure, and the blue circle is the outcome.

Table S2. Study sample characteristics by time-point. The Tromsø Study 1986– 2001.

			T1 1986/87		**T2 1994/95**		**T3 2001**	
			Mean (SD)	**ΔMean case-control (95% CI)**	**Mean (SD)**	**ΔMean case-control (95% CI)**	**Mean (SD)**	**ΔMean case-control (95% CI)**
Age (years)	Women	Case	46.3 (6.36)	2.50 (-0.19, 5.19)	54.3 (6.36)	2.50 (-0.19, 5.19)	61.3 (6.36)	2.50 (-0.19, 5.19)
		Control	43.8 (8.88)		51.8 (8.88)		58.8 (8.88)	
	Men	Case	48.8 (8.66)	2.09 (-1.49, 5.64)	56.8 (8.66)	2.09 (-1.49, 5.64)	63.8 (8.66)	2.09 (-1.49, 5.64)
		Control	46.7 (10.7)		54.7 (10.7)		61.7 (10.7)	
BMI (kg/m^2)	Women	Case	27.1 (4.27)	3.35** (1.97, 4.72)	29.2 (4.91)	4.21** (2.58, 5.83)	31.2 (5.69)	4.88** (3.12, 6.63)
		Control	23.7 (3.75)		25.0 (4.61)		26.4 (4.60)	
	Men	Case	27.6 (3.49)	2.92** (1.79, 4.05)	28.7 (3.44)	3.01** (1.84, 4.18)	29.8 (3.52)	3.28** (2.02, 4.55)
		Control	24.7 (2.72)		25.7 (3.02)		26.6 (3.44)	
Menarche age (years)	Women	Case	13.4 (1.26)	-0.02 (-0.46, 0.50)	13.4 (1.26)	-0.02 (-0.46, 0.50)	13.4 (1.26)	-0.02 (-0.46, 0.50)
		Control	13.3 (1.50)		13.3 (1.35)		13.4 (1.55)	
Parity (n)	Women	Case	2.79 (0.20)	0.39 (-0.15, 0.93)	2.93 (0.19)	0.41 (-0.10, 0.92)	2.95 (0.20)	0.33 (-0.18, 0.85)
		Control	2.40 (0.18)		2.55 (0.18)		2.61 (0.17)	
			n (%)	***p*-value**	**n (%)**	***p*-value**	**n (%)**	***p*-value**
Physical activity: Women	Active	Case	48 (80.0)	0.71	26 (43.3)	<0.01	44 (74.6)	0.64
		Control	58 (77.3)		51 (68.0)		57 (78.1)	
	Inactive	Case	12 (20.0)		34 (56.7)		15 (25.4)	
		Control	17 (22.7)		24 (32.0)		16 (21.9)	
Physical activity: Men	Active	Case	46 (82.1)	0.96	39 (69.6)	0.55	42 (77.8)	0.62
		Control	52 (82.5)		47 (74.6)		45 (73.8)	
	Inactive	Case	10 (17.9)		17 (30.4)		12 (22.2)	
		Control	11 (17.5)		16 (25.4)		16 (26.2)	
Menstrual status: Women	Yes	Case	35 (58.3)	0.85	12 (20.0)	0.08	5 (8.3)	0.16
		Control	42 (56.0)		25 (33.3)		7 (9.3)	
	No	Case	21 (35.0)		48 (80.0)		54 (90.0)	
		Control	26 (34.7)		48 (64.0)		61 (81.3)	
	Uncertain/ Irregular	Case	4 (6.7)		0 (0.0)		1 (1.7)	
		Control	7 (9.3)		2 (2.7)		7 (5.3)	
Contraceptives: Women	No	Case	25 (43.9)	0.82	28 (56.0)	0.52	39 (68.4)	0.83
		Control	31 (41.9)		32 (50.0)		50 (66.7)	
	Yes	Case	32 (56.1)		28 (56.0)		18 (31.6)	
		Control	43 (58.1)		32 (50.0)		25 (33.3)	
Hormone replacement treatment: Women	No	Case	54 (96.4)	0.80	41 (74.6)	0.20	29 (50.0)	0.78

		Control	70 (97.2)		42 (60.0)		34 (45.3)	
	Yes	Case	2 (3.6)		11 (20.0)		22 (37.9)	
		Control	2 (2.8)		24 (34.3)		33 (44.0)	
	Previously	Case	--		3 (5.5)		7 (12.1)	
		Control	--		4 (5.7)		8 (10.7)	
Elevated blood pressure: Women	No	Case	22 (36.7)	<0.01	17 (28.3)	0.02	10 (16.7)	<0.01
		Control	50 (66.7)		36 (48.0)		32 (42.7)	
	Yes	Case	38 (63.3)		43 (71.7)		50 (83.3)	
		Control	25 (33.3)		39 (52.0)		43 (57.3)	
Elevated blood pressure: Men	No	Case	13 (23.2)	0.22	11 (19.6)	0.45	11 (19.6)	0.73
		Control	21 (33.3)		16 (25.4)		14 (22.2)	
	Yes	Case	43 (76.8)		45 (80.4)		45 (80.4)	
		Control	42 (66.7)		47 (74.6)		49 (77.8)	
Family history of type 2 diabetes: Women	No	Case	45 (75.0)	0.03	38 (63.3)	<0.01	35 (58.3)	0.01
		Control	67 (89.3)		65 (86.7)		60 (80.0)	
	Yes	Case	15 (25.0)		22 (36.7)		25 (41.7)	
		Control	8 (10.7)		10 (13.3)		15 (20.0)	
Family history of type 2 diabetes: Men	No	Case	45 (80.4)	0.59	42 (75.0)	0.57	39 (69.6)	0.42
		Control	53 (84.1)		50 (79.4)		48 (76.2)	
	Yes	Case	11 (19.6)		14 (25.0)		17 (30.4)	
		Control	10 (15.9)		13 (20.6)		15 (23.8)	

Sample numbers at each time point: women: 60 cases and 75 controls; men: 56 cases and 63 controls. BMI, body mass index; CI, confidence interval; SD, standard deviation; T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

Table S3. Androgen concentrations at all time-points in men and women comparing cases and controls. Results presented as median with IQR. The Tromsø Study 1986–2001.

			Pre-diagnostic time-points					
			T1 1986/87		**T2 1994/95**		**T3 2001**	
Hormone			**Median (IQR)**	**p-value**	**Median (IQR)**	**p-value**	**Median (IQR)**	**p-value**
11OHT (nmol/l)	Women[a]	Case	0.47 (0.34)	0.13	0.51 (0.43)	<0.01	0.53 (0.41)	0.01
		Control	0.43 (0.35)		0.44 (0.28)		0.44 (0.39)	
	Men[b]	Case	0.67 (0.34)	<0.01	0.60 (0.41)	<0.01	0.67 (0.51)	<0.01
		Control	0.45 (0.45)		0.41 (0.27)		0.47 (0.32)	
11OHA4 (nmol/l)	Women	Case	3.94 (2.71)	0.98	3.98 (2.74)	0.10	4.21 (2.84)	0.22
		Control	3.83 (3.08)		3.63 (2.56)		4.06 (2.76)	
	Men	Case	5.60 (3.00)	0.04	4.68 (3,17)	0.01	5.29 (2,81)	<0.01
		Control	4.44 (2.80)		3.53 (2.80)		4.24 (2.69)	
A4 (nmol/l)	Women	Case	2.63 (1.83)	0.28	1.71 (1.31)	0.96	1.95 (1,47)	0.85
		Control	2.75 (2.18)		1.79 (1,28)		2.00 (1.36)	
	Men	Case	2.39 (1.50)	0.60	1.84 (0.97)	0.79	2.25 (1.22)	0.71
		Control	2.53 (1.23)		1.90 (0.99)		2.22 (1.03)	
11KA4 (nmol/l)	Women	Case	0.48 (0.30)	0.34	0.40 (0,31)	0.39	0.55 (0.45)	0.33
		Control	0.53 (0,46)		0.41 (0.30)		0.50 (0.37)	
	Men	Case	0.65 (0.36)	0.01	0.47 (0.30)	0.02	0.65 (0.44)	<0.01
		Control	0.53 (0,41)		0.36 (0.24)		0.57 (0.34)	
DHEAS (µmol/l)	Women	Case	3.52 (2.83)	0.90	2.30 (2.55)	0.77	2.00 (2.23)	0.96
		Control	3.55 (3.24)		2.32 (2.00)		2.02 (2.38)	
	Men	Case	5.40 (3.08)	0.46	4.29 (2.61)	0.53	3.37 (1.99)	0.56
		Control	6.05 (3.64)		3.86 (3.24)		3.18 (3.79)	

11KT (nmol/l)	Women	Case	1.22 (0.94)	0.97	1.13 (0.72)	0.09	1.27 (0.82)	0.06
		Control	1.23 (0,87)		1.02 (0.62)		1.10 (0.79)	
	Men	Case	1.48 (0.85)	<0.01	1.30 (0.75)	<0.01	1.45 (0.85)	<0.01
		Control	1.13 (0.90)		0.92 (0.54)		1.03 (0.79)	
Testosterone (nmol/l)	Women	Case	0.69 (0.34)	0.61	0.73 (0.43)	0.28	0.69 (0.39)	0.58
		Control	0.67 (0.45)		0.80 (0.52)		0.68 (0.46)	
	Men	Case	15.5 (8.31)	0.01	14.5 (6.71)	<0.01	13.3 (6.30)	<0.01
		Control	17.9 (8.52)		16.9 (9.28)		16.7 (8.91)	

Sample numbers at each time point: women: 60 cases and 75 controls; men: 56 cases and 63 controls. 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrostenedione; A4, androstenedione; DHEAS, dehydroepiandrosterone sulphate; IQR, inter quartile range; T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

Table S4. Androgen concentrations at all time-points, adjusted for confounders[a]. Models were generalised estimating equations with log-link and gamma distribution. Androgen concentrations were the dependent variable with time-points and confounders as independent variables. T3 is set as the reference time-point. The Tromsø Study 1986–2001.

Biomarker		Men			Women		
		β-coefficient (SE)	p-value	95% confidence interval	β-coefficient (SE)	p-value	95% confidence interval
11OHT (nmol/l)	Case	0.35 (0.12)	<0.01	0.12, 0.58	0.08 (0.10)	0.45	-0.13, 0.28
	T1	0.10 (0.14)	0.49	-0.18, 0.37	0.10 (0.12)	0.39	-0.13, 0.33
	T2	-0.05 (0.09)	0.59	-0.24, 0.13	-0.01 (0.07)	0.86	-0.16, 0.13
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	-0.16 (0.12)	0.17	-0.39, 0.07	-0.17 (0.11)	0.13	-0.39, 0.05
	Case#T2	-0.07 (0.11)	0.55	-0.29, 0.15	0.06 (0.09)	0.53	-0.12, 0.24
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	-1.55 (0.55)	0.01	-2.63, -0.47	-1.95 (0.44)	<0.01	-2.81, -1.08
Adjusted cases compared to controls at single time-points	T1	0.19 (0.12)	0.11	-0.04, 0.42	-0.09 (0.09)	0.34	-0.28, 0.10
	T2	0.28 (0.12)	0.02	0.05, 0.51	0.14 (0.09)	0.13	-0.04, 0.32
	T3	0.35 (0.12)	<0.01	0.12, 0.58	0.08 (0.10)	0.45	-0.13, 0.28
11OHA4 (nmol/l)	Case	0.23 (0.09)	0.02	0.04, 0.41	0.03 (0.09)	0.71	-0.15, 0.22
	T1	0.15 (0.11)	0.17	-0.06, 0.35	0.05 (0.09)	0.56	-0.12, 0.23
	T2	-0.12 (0.06)	0.06	-0.24, 0.01	-0.13 (0.09)	0.04	-0.26, -0.004
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	-0.12 (0.11)	0.27	-0.33, 0.09	-0.10 (0.10)	0.35	-0.30, 0.11
	Case#T2	-0.05 (0.09)	0.56	-0.23, 0.13	0.07 (0.08)	0.43	-0.10, 0.23
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	1.22 (0.46)	0.01	0.32, 2.12	0.82 (0.41)	0.05	0.02, 1.63
Adjusted cases compared to controls at single time-points	T1	0.11 (0.10)	0.27	-0.08, 0.30	-0.05 (0.09)	0.50	-0.24, 0.12
	T2	0.17 (0.09)	0.06	-0.004, 0.35	0.10 (0.09)	0.25	-0.07, 0.27
	T3	0.23 (0.09)	0.02	0.04, 0.41	0.03 (0.09)	0.71	-0.15, 0.22
A4 (nmol/l)	Case	0.01 (0.08)	0.89	-0.14, 0.16	0.10 (0.09)	0.28	-0.08, 0.28
	T1	0.10 (0.12)	0.41	-0.14, 0.35	-0.15 (0.07)	0.04	-0.30, -0.01
	T2	-0.18 (0.05)	<0.01	-0.29, -0.08	-0.30 (0.05)	<0.01	-0.41, -0.19
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	-0.06 (0.10)	0.52	-0.19, 0.13	-0.12 (0.10)	0.25	-0.33, 0.09
	Case#T2	-0.05 (0.08)	0.54	-0.19, 0.10	-0.03 (0.08)	0.74	-0.19, 0.13
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	1.53 (0.43)	<0.01	0.68, 2.38	1.73 (0.31)	<0.01	1.12, 2.35
Adjusted cases compared to controls at single time-points	T1	-0.05 (0.10)	0.62	-0.25, 0.15	-0.02 (0.07)	0.80	-0.17, 0.13
	T2	-0.04 (0.07)	0.62	-0.17, 0.10	0.07 (0.08)	0.39	-0.09, 0.24
	T3	0.01 (0.08)	0.89	-0.14, 0.16	0.10 (0.09)	0.28	-0.08, 0.28
11KA4 (nmol/l)	Case	0.25 (0.10)	0.01	0.05, 0.45	-0.01 (0.10)	0.93	-0.20, 0.18
	T1	-0.04 (0.10)	0.68	-0.24, 0.16	-0.10 (0.08)	0.25	-0.26, 0.07
	T2	-0.41 (0.06)	<0.01	-0.53, -0.29	-0.36 (0.07)	<0.01	-0.49, -0.22
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	-0.05 (0.11)	0.62	-0.26, 0.15	-0.11 (0.10)	0.28	-0.30, 0.09
	Case#T2	-0.06 (0.10)	0.53	-0.26, 0.13	-0.003 (0.09)	0.97	-0.18, 0.17
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	-0.20 (0.53)	0.70	-1.24, 0.83	-0.31 (0.42)	0.46	-1.14, 0.51
Adjusted cases compared to controls at single time-points	T1	0.20 (0.10)	0.04	0.01, 0.39	-0.11 (0.09)	0.22	-0.30, 0.07
	T2	0.19 (0.10)	0.06	-0.01, 0.38	-0.01 (0.09)	0.89	-0.18, 0.16
	T3	0.25 (0.10)	0.01	0.05, 0.45	-0.01 (0.10)	0.93	-0.20, 0.18
DHEAS (µmol/l)	Case	-0.08 (0.11)	0.49	-0.29, 0.14	0.09 (0.10)	0.40	-0.12, 0.29
	T1	0.03 (0.08)	0.74	-0.13, 0.18	-0.21 (0.09)	0.01	-0.38, -0.05
	T2	-0.08 (0.05)	0.09	-0.18, 0.01	-0.19 (0.05)	<0.01	-0.28, -0.09
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	0.07 (0.08)	0.40	-0.09, 0.22	0.03 (0.08)	0.71	-0.13, 0.20
	Case#T2	0.05 (0.06)	0.39	-0.07, 0.18	0.06 (0.08)	0.45	-0.09, 0.20
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	9.87 (0.39)	<0.01	9.11, 10.6	2.24 (0.46)	<0.01	1.34, 3.14
Adjusted cases compared to controls at single time-points	T1	-0.01 (0.08)	0.89	-0.17, 0.15	0.12 (0.09)	0.17	-0.05, 0.29
	T2	-0.02 (0.09)	0.80	-0.19, 0.15	0.14 (0.09)	012	-0.04, 0.32
	T3	-0.08 (0.11)	0.49	-0.29, 0.14	0.09 (0.10)	0.40	-0.12, 0.29

11KT (nmol/l)	Case	0.22 (0.09)	0.02	0.04, 0.41	0.08 (0.10)	0.43	-0.11, 0.27
	T1	0.06 (0.10)	0.58	-0.14, 0.25	0.10 (0.09)	0.25	-0.07, 0.28
	T2	-0.14 (0.06)	0.03	-0.26, -0.01	-0.06 (0.06)	0.35	-0.18, 0.06
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	-0.03 (0.10)	0.79	-0.22, 0.17	-0.16 (0.09)	0.08	-0.34, 0.02
	Case#T2	-0.03 (0.08)	0.70	-0.19, 0.13	0.002 (0.09)	0.98	-0.17, 0.17
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	-0.42 (0.38)	0.27	-1.16, 0.33	-0.33 (0.46)	0.47	-1.24, 0.57
Adjusted cases compared to controls at single time-points	T1	0.19 (0.10)	0.04	0.01, 0.38	-0.08 (0.09)	0.35	-0.26, 0.09
	T2	0.19 (0.10)	0.05	0.002, 0.38	0.08 (0.09)	0.40	-0.10, 0.26
	T3	0.22 (0.09)	0.02	0.04, 0.41	0.08 (0.10)	0.43	-0.11, 0.27
Testosterone (nmol/l)	Case	-0.11 (0.06)	0.09	-0.23, 0.02	-0.05 (0.10)	0.63	-0.24, 0.14
	T1	0.02 (0.07)	0.81	-0.12, 0.15	-0.02 (0.10)	0.81	-0.22, 0.17
	T2	0.02 (0.04)	0.65	-0.07, 0.11	0.45 (0.31)	0.14	-0.15, 1.05
	T3	*Reference*	--	--	*Reference*	--	--
	Case#T1	0.02 (0.06)	0.80	-0.10, 0.13	-0.07 (0.10)	0.52	-0.27, 0.14
	Case#T2	-0.002 (0.06)	0.98	-0.12, 0.11	-0.43 (0.28)	0.12	-0.97, 0.11
	Case#T3	*Reference*	--	--	*Reference*	--	--
	Constant	3.94 (0.36)	<0.01	3.24, 4.64	-1.47 (0.71)	0.04	-2.86, -0.09
Adjusted cases compared to controls at single time-points	T1	-0.09 (0.06)	0.15	-0.22, 0.03	-0.11 (0.08)	0.15	-0.27, 0.04
	T2	-0.11 (0.06)	0.09	-0.23, 0.02	-0.47 (0.31)	0.13	-1.08, 0.14
	T3	-0.11 (0.06)	0.09	-0.23, 0.02	0.05 (0.10)	0.63	-0.24, 0.14

[a]Adjusted for age, body mass index, and physical activity, and additionally for age at menarche, parity, menstrual status, and use of oral/intrauterine contraceptives for women.

Sample numbers at each time point: women: 60 cases and 75 controls; men: 56 cases and 63 controls.

11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrostenedione; A4, androstenedione; DHEAS, dehydroepiandrosterone; SE, standard error; T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).

Table S5. Crude and adjusted[a] ORs of type 2 diabetes for each androgen by time-point and sex. The Tromsø Study 1986– 2001.

		T1 (1986/87)		T2 (1994/95)		T3 (2001)	
		Crude	Adjusted[a]	Crude	Adjusted[a]	Crude	Adjusted[a]
Biomarker	**Sex**	**OR (95%CI)**	**OR (95%CI)**	**OR (95%CI)**	**OR (95%CI)**	**OR (95%CI)**	**OR (95%CI)**
11OHT (nmol/l)	Women	1.16 (0.75, 1.78)	0.70 (0.41, 1.20)	2.20 (1.34, 3.63)	1.67 (0.80, 3.46)	1.61 (1.11, 2.34)	1.23 (0.78, 1.93)
	Men	1.63 (1.12, 2.38)	1.42 (0.92, 2.17)	2.09 (1.32, 3.32)	1.56 (0.99, 2.44)	2.16 (1.40, 3.32)	1.76 (1.12, 2.75)
11OHA4 (nmol/l)	Women	0.97 (0.62, 1.52)	0.78 (0.44, 1.38)	1.63 (0.99, 2.68)	1.39 (0.62, 3.13)	1.34 (0.89, 2.02)	1.03 (0.60, 1.76)
	Men	1.36 (0.94, 1.97)	1.30 (0.86, 1.97)	1.83 (1.11, 3.04)	1.57 (0.90, 2.72)	2.25 (1.33, 3.82)	2.00 (1.12, 3.56)
A4 (nmol/l)	Women	0.84 (0.54, 1.31)	1.02 (0.52, 1.97)	0.89 (0.50, 1.57)	1.35 (0.47, 3.90)	0.99 (0.58, 1.70)	0.99 (0.47, 2.10)
	Men	0.90 (0.69, 1.18)	0.92 (0.66, 1.27)	0.80 (0.51, 1.28)	1.00 (0.56, 1.78)	0.92 (0.60, 1.40)	1.00 (0.61, 1.63)
11KA4 (nmol/l)	Women	0.87 (0.56, 1.34)	0.71 (0.41, 1.22)	1.28 (0.72, 2.25)	0.92 (0.36, 2.39)	1.21 (0.82, 1.77)	0.92 (0.55, 1.53)
	Men	1.52 (1.03, 2.24)	1.59 (1.02, 2.49)	1.54 (0.90, 2.61)	1.64 (0.92, 2.92)	2.03 (1.26, 3.27)	1.84 (1.09, 3.10)
DHEAS (µmol/l)	Women	1.00 (0.67, 1.45)	1.35 (0.76, 2.42)	1.07 (0.69, 1.65)	1.94 (0.79, 4.76)	0.93 (0.61, 1.44)	1.22 (0.68, 2.20)
	Men	0.80 (0.46, 1.42)	0.95 (0.45, 2.04)	0.72 (0.38, 1.36)	0.83 (0.35, 1.97)	0.63 (0.33, 1.22)	0.65 (0.29, 1.46)
11KT (nmol/l)	Women	1.04 (0.68, 1.58)	0.74 (0.43, 1.28)	1.63 (1.01, 2.63)	1.23 (0.60, 2.53)	1.52 (1.02, 2.25)	1.30 (0.80, 2.10)
	Men	1.85 (1.24, 2.78)	1.62 (1.02, 2.57)	2.15 (1.28, 3.61)	1.64 (0.94, 2.86)	2.19 (1.36, 3.51)	1.78 (1.03, 3.08)
Testosteron (nmol/l)	Women	0.86 (0.54, 1.39)	0.83 (0.44, 1.58)	0.99 (0.93, 1.06)	0.65 (0.33, 1.29)	1.14 (0.78, 1.68)	0.99 (0.62, 1.60)
	Men	0.41 (0.23, 0.75)	0.60 (0.31, 1.19)	0.36 (0.19, 0.67)	0.52 (0.25, 1.08)	0.32 (0.16, 0.63)	0.45 (0.21, 0.98)

[a]Adjusted for age, body mass index, and physical activity, and additionally age at menarche, parity, menstrual status, and use of oral/intrauterine contraceptives for women.

Odds ratios for all androgens are estimated per 1-IQR increase. Sample numbers at each time point: women: 60 cases and 75 controls; men: 56 cases and 63 controls. 11KA4, 11-ketoandrostenedione; 11KT, 11-ketotestosterone; 11OHT, 11-hydroxytestosterone; 11OHA4, 11-hydroxyandrostenedione; A4, androstenedione; CI, confidence interval; DHEAS, dehydroepiandrosterone sulphate; IQR, interquartile range; OR, odds ratio; T1, Tromsø3 (1986/87); T2, Tromsø4 (1994/95); T3, Tromsø5 (2001).