SUPPLEMENTARY DATA

It is unclear whether elevated transferrin saturation levels, a biochemical marker useful in the diagnosis of iron overload syndromes such as hemochromatosis, confer increased risk of common forms of diabetes mellitus in the general population. This question may have important public health consequences, since oxidative stress has been implicated in the pathogenesis of both insulin resistance and inflammatory beta-cell destruction in common diabetes, since iron overload is preventable by dietary restrictions, and because the diabetes is in part reversible after reduction of iron overload(1-3). Most investigations of individuals with iron overload(4-12) or with the C282Y/C282Ygentope(13,14) reporting positive associations with diabetes have been case-control studies; further, with few exceptions(11,14), previous studies have focused on Type 2 diabetes or any diabetes(4-9,13).

Research Design and Methods

General population studies

Copenhagen City Heart Study (CCHS): This is a prospective study of the Danish general population initiated in 1976-1978 (n=14223), with follow-up examinations in 1981-1983 (n=12698), 1991-1994 (n=10135), and 2001-2003 (n=6238)(15); DNA was isolated in most of those attending the 1991-1994 (n=9259) and/or 2001-2003 examinations (additional n=1352), some of whom also participated in the 1976-78 (n=6865) and 1981-83 examinations (n=7084). Individuals were randomly selected based on the national Danish Civil Registration System to reflect the Copenhagen general population aged 20-80+ years. Of 9259 giving blood for DNA from the third examination of CCHS in 1991 to 1994, we examined 9121 individuals (5164 women and 3957 men) with eligible transferrin saturation. Median age was 60 years (interquartile range: 48 to 70 years). Response rate was 61%.

The Copenhagen General Population Study (CGPS): This is prospective study of the Danish general population initiated in 2003 and still recruiting; the aim is to total 100,000 participants ascertained exactly like in the Copenhagen City Heart Study, but from a different part of Copenhagen(16). Individuals were randomly selected based on the national

Danish Civil Registration System to reflect the adult Copenhagen general population aged 20-80+ years. At the time of the present study, we included individuals up until 2007; response rate is 45%. We included the first 24195 responders (13079 women and 11116 men) with a median age of 59 years (interquartile range: 49 to 68 years). All individuals had eligible transferrin saturation.

Population-based case-control study

We recruited consecutively population-based patients with diabetes (N=6129, 2673 women and 3456 men) with a median age of 56 years (interquartile range: 42 to 66 years) from Copenhagen County who attended the Steno Diabetes Centre between November 2001 and November 2007. All individuals had eligible transferrin saturation. None of the patients were diagnosed with hemochromatosis at recruitment. Age and gender matched controls (N=6129) were collected as in the CGPS and all recruited from Copenhagen County similarly to the cases. Subjects in the case-control study were different than those included in the CGPS study. **Steno Diabetes Centre is a first-line referral clinic for Type 1 diabetes in Copenhagen, and is representative of a population of 600,000 inhabitants; more than 90% of those diagnosed with Type 1 diabetes is referred to the centre(14).**

Other measurements

In CCHS and CGPS, plasma haemoglobin concentration was measured by spectrophotometry (Konelab autoanalyzer (ThermoFisher Scientific)), and body mass index (BMI) calculated as

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weight in kilograms divided by height in meters squared. These data were not available for the population-based case-control study.

Genotyping

Individuals from the general population studies and patients with diabetes from Steno Diabetes Center were genotyped. Genotyping of the CCHS and patients with diabetes for C282Y (dbSNP:rs1800562), a G/A nucleotide change at position 845 in the HFE gene(17), and H63D (dbSNP:rs1799945), a C/G nucleotide change at position 187 in the HFE gene(17), was by allele specific amplification(18), and restriction enzyme digestion to confirm genotyping(14,17). The amplification refractory mutation system (ARMS) simultaneously detects both hereditary hemochromatosis mutations C282Y and H63D including sense and antisense primers for C282Y, H63D, and human growth hormone as internal amplification control(18). Genotyping of the CGPS was by a TaqMan assay (Applied Biosystems, Foster City, Calif) (details available from authors), confirmed using sequencing.

Diabetes mellitus endpoint definitions

General population studies:

Individuals were observed from 1976 (when the national Danish Patient Registry began) until event, death, emigration or May 2009; thus follow-up was 33 years. A combination of ICD-codes (Type 1 diabetes (ICD8: 249, ICD10: E10), and Type 2 or other or unspecified diabetes (ICD8:250, ICD10: E11, E13, E14)) from the National Danish Patient Registry and the National Danish Causes of Death Registry from 1976 and onwards, self-reported diabetes mellitus (yes/no), information on anti-diabetic medication, and a non-fasting glucose above 11 mmol/L was used. In CCHS and CGPS, 910 and 1496 were classified with diabetes mellitus. 274 and 393 were classified with Type 1 diabetes, and 875 and 1418 were classified with Type 2 diabetes or other or unspecified diabetes mellitus, respectively. However, in the National Danish Patient Registry, 239 and 315 had diagnoses for both Type 1 and Type 2 diabetes in CCHS and CGPS, respectively; thus the stratification on Type 1 and Type 2 for the general population studies is not mutually exclusive. As CGPS started recruitment 10 years later than CCHS, the number of participants developing diabetes in CGPS was only 1.4 times that in CCHS, even though CGPS was 2.6 times larger than CCHS.

Population-based case-control study:

ICD10-codes (E10 to E14) from the National Danish Patient Registry were used as described above. Patients were classified with Type 1 diabetes (E10) (N=2664) and with Type 2 diabetes (E11) (N=3177) or other or unspecified diabetes mellitus,(E13, E14) (N=288) (Total N=3465).

Since the Steno Diabetes Center is a first-line referral clinic for Type 1 diabetes in Copenhagen County, this sample is representative of a population of about 600,000 inhabitants. The patients with Type 2 diabetes comprise first-line referrals combined with referrals from departments of Nephrology and Endocrinology due to diabetic complications and start of insulin therapy. All patients had a blood test for transferrin saturation measurement at the first meeting with the endocrinologist. There were no duplicate individuals between this study and our previous study(14).

Statistics

STATA/SE 10.0 statistical software package was used. Two-sided $P \leq 0.05$ was considered significant. In the general population studies, cumulative incidence rates of diabetes mellitus were plotted with the use

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of Kaplan-Meier curves and differences between transferrin saturation levels were examined by log-rank tests and stratified for body mass index (BMI).

To obtain maximal statistical power, combined risks from the CCHS, the CGPS, and the case-control study were calculated in a meta-analysis as fixed and random-effect measures using Mantel-Haentzel statistics. In the fixed-effect model, we assumed that all studies were carried out in a common population and the effect size was not significantly different among the studies; in the random-effects model, we incorporated the random variation within the studies and the variation between the different studies. Statistical heterogeneity was assessed using Q-statistics (acknowledging that the number of studies only equalled three)(19).

Population attributable risk was estimated as [f(risk-1)]/[1-f(risk-1)], where f is the frequency of transferrin saturation ≥50% in the population, and risk is either the hazard ratio or odds ratio for diabetes mellitus(20).

Results

Characteristics of participants in the two general population studies and the population-based case-control study are shown in Online-Only Table 1. The study comprised 8535 patients with diabetes (910 from the CCHS, 1496 from the CGPS, and 6129 from the population-based case-control study), and 37039 individuals without diabetes from the general population (8211 from the CCHS, 22699 from the CGPS, and 6129 from the population-based case-control study).

Risk of any diabetes mellitus

The cumulative incidence by age of diabetes mellitus was increased in individuals with a transferrin saturation ≥50% versus <50% in the CCHS (log-rank: p=0.0001) (Online-Only Figure1A) and the CGPS (p=0.03) (Online-Only Figure1B) with risk beginning to increase at the age of approximately 50 years.

Population attributable risk

A population attributable risk illustrates how much the risk of diabetes mellitus would be reduced in the general population if transferrin saturation ≥50% was not present. Based on a prevalence of 4% for transferrin saturation ≥50% in the CCHS and a hazard ratio of 1.8 for any diabetes, the population attributable risk was 3%. Based on a prevalence of 1.5% for transferrin saturation ≥50% in the CGPS and a hazard ratio of 1.4 for any diabetes, the population attributable risk was 1%. Finally, based on a frequency of 3% for transferrin saturation ≥50% in the population-based case-control study and an odds ratio of 3.3 for any diabetes, the population attributable risk was 7%. The combined population attributable risk for the two general population studies and the population-based case-control study was 3%. Thus, 25, 15, and 400 individuals with diabetes in the CCHS, the CGPS, and at the Steno Diabetes Center could have been avoided if they had not had elevated transferrin saturation.

The corresponding population attributable risks for Type 1 diabetes were 4% for the CCHS, 1% for the CGPS, 22% for the population-based case-control study, and 3% combined. Likewise, the corresponding population attributable risks for Type 2 diabetes were 3% for the CCHS, 1% for the CGPS, 1% for the population-based case-control study, and 1% combined.

Hemochromatosis genotypes among individuals with elevated transferrin saturation

The genotype distribution was not statistically different (p=0.08) in individuals with diabetes in the general population studies versus Steno Diabetes Center or in individuals with diabetes vs. no diabetes

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in the general population studies only (Online-only Table 2); thus the risk of increased transferrin saturation observed in our study is not secondary to an increased risk conferred by the genotype.

Conclusions

The risk of increased transferrin saturation observed in our study was not secondary to an increased risk conferred by the C282Y or H63D genotype. **Other genetic factors (not investigated in this study) that are major determinants of serum iron and transferrin saturation in the general population, could potentially also influence our results(21,22), and an increased risk of transferrin saturation could theoretically also be secondary to diabetes.**

Diabetes as a consequence of hemochromatosis is characterised by both insulin resistance and beta-cell destruction(23), and the metabolic phenotype can therefore mimic both Type 2 diabetes and non-autoimmune Type 1 diabetes. The defects in both insulin-producing and insulin-sensitive tissues are most likely caused by iron-dependent catalysis via the Fenton reaction of reactive oxygen radical species, which impair insulin signalling in skeletal muscle and liver and cause beta-cell destruction due to insufficient beta-cell deficient antioxidant defence(24), with main emphasis on beta-cell destruction in Type 1 diabetes and insulin resistance in Type 2 diabetes(25).

Strengths of our work include the fact that the patients with diabetes was a large group, representative, and well defined, thereby reducing the possibility of bias. The patients in the case-control study almost exclusively came from Copenhagen County, and therefore formed an unselected representative group. Misclassification of the subjects in the case-control study is unlikely, since general practitioners diagnosed the patients and then referred them to Steno Diabetes Center, a highly sub-specialised diabetes-clinic. The strength of the general population studies was that they were large and subjects sampled randomly.

Limitations in our study were that the sub-classification of diabetes was not as well-defined in the general population studies as in the case-control study and that we studied Caucasians only and therefore our results may not apply to other races. **Also, the meta-analytical approach to include the three study populations to maximize statistical power is open to selection bias.**

We cannot exclude that some individuals with high transferrin saturation could have a bone marrow disorder with reduced or high iron turnover such as hypoplastic anemia or haemolytic disease, hepatocellular injury, or a transient non-specific rise in transferrin saturation rather than increased iron stores; however, a significant contribution such of conditions is unlikely because of the rarity of these diseases.

In context of existing literature three other studies(8,9,11) have used transferrin saturation as the primary independent variable (without considering hemochromatosis genotype) for the study of the risk of diabetes. However, these studies are heterogeneous concerning ethnicity (two of the studies use mixed populations(8,9), another doesn't report ethnicity(11)), study design (cross-sectional(9), retrospective cohort(8), and case-study with historical controls(11)), size (44000 Whites/53000 other ethnicities(9), 9000(8), and 820(11)), transferrin saturation threshold (no threshold but only mean values(9), threshold of at least 45%(8), threshold of 35%(11)), diabetes diagnosis (any diabetes(8,9), Type 1(11), Type 2(11)), source of diabetes diagnosis (self-report(8,9), use of ICD-9 code(8) for diabetes patients in an endocrinology department(11)), and finally also results (one in favour(11) and two not infavour(8,9) of association of elevated transferrin saturation and risk of diabetes). In comparison, our study consisted of three individual studies consistent in study design (two large general population studies ascertained alike, and a case-control study using patients with diabetes ascertained from a similar population as the general population studies and compared to controls ascertained like the population studies with no overlap of individuals in the three individual studies), ethnicity (only White individuals), transferrin saturation threshold value of 50% in accordance with accepted clinical

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practice(26-28), diabetes diagnosis (any diabetes, Type 1 and Type 2 for all three studies), source of the diabetes diagnosis (self-report, medication, ICD-coding, and a non-fasting glucose above 11 mmol/L), and finally also consistent in results.

In conclusion, transferrin saturation ≥50% was associated with 2-3 fold increased risk of any diabetes mellitus, Type 1 diabetes, and Type 2 diabetes, independently of hemochromatosis genotype.

It is unclear whether elevated transferrin saturation levels, a biochemical marker useful in the diagnosis of iron overload syndromes such as hemochromatosis, confer increased risk of common forms of diabetes mellitus in the general population. This question may have important public health consequences, since oxidative stress has been implicated in the pathogenesis of both insulin resistance and inflammatory beta-cell destruction in common diabetes, since iron overload is preventable by dietary restrictions, and because the diabetes is in part reversible after reduction of iron overload(1-3). Most investigations of individuals with iron overload(4-12) or with the C282Y/C282Ygentope(13,14) reporting positive associations with diabetes have been case-control studies; further, with few exceptions(11,14), previous studies have focused on Type 2 diabetes or any diabetes(4-9,13).

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diabetes in Copenhagen, and is representative of a population of 600,000 inhabitants; more than 90% of those diagnosed with Type 1 diabetes is referred to the centre(14).

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for transferrin saturation measurement at the first meeting with the endocrinologist. There were no duplicate individuals between this study and our previous study(14).

Statistics

STATA/SE 10.0 statistical software package was used. Two-sided P≤0.05 was considered significant. In the general population studies, cumulative incidence rates of diabetes mellitus were plotted with the use of Kaplan-Meier curves and differences between transferrin saturation levels were examined by log-rank tests and stratified for body mass index (BMI).

To obtain maximal statistical power, combined risks from the CCHS, the CGPS, and the case-control study were calculated in a meta-analysis as fixed and random-effect measures using Mantel-Haentzel statistics. In the fixed-effect model, we assumed that all studies were carried out in a common population and the effect size was not significantly different among the studies; in the random-effects model, we incorporated the random variation within the studies and the variation between the different studies. Statistical heterogeneity was assessed using Q-statistics (acknowledging that the number of studies only equalled three)(19).

Population attributable risk was estimated as [f(risk-1)]/[1-f(risk-1)], where f is the frequency of transferrin saturation ≥50% in the population, and risk is either the hazard ratio or odds ratio for diabetes mellitus(20).

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Characteristics of participants in the two general population studies and the population-based case-control study are shown in Online-Only Table 1. The study comprised 8535 patients with diabetes (910 from the CCHS, 1496 from the CGPS, and 6129 from the population-based case-control study), and 37039 individuals without diabetes from the general population (8211 from the CCHS, 22699 from the CGPS, and 6129 from the population-based case-control study).

Risk of any diabetes mellitus

The cumulative incidence by age of diabetes mellitus was increased in individuals with a transferrin saturation ≥50% versus <50% in the CCHS (log-rank: p=0.0001) (Online-Only Figure1A) and the CGPS (p=0.03) (Online-Only Figure1B) with risk beginning to increase at the age of approximately 50 years.

Population attributable risk

A population attributable risk illustrates how much the risk of diabetes mellitus would be reduced in the general population if transferrin saturation ≥50% was not present. Based on a prevalence of 4% for transferrin saturation ≥50% in the CCHS and a hazard ratio of 1.8 for any diabetes, the population attributable risk was 3%. Based on a prevalence of 1.5% for transferrin saturation ≥50% in the CGPS and a hazard ratio of 1.4 for any diabetes, the population attributable risk was 1%. Finally, based on a frequency of 3% for transferrin saturation ≥50% in the population-based case-control study and an odds ratio of 3.3 for any diabetes, the population attributable risk was 7%. The combined population attributable risk for the two general population studies and the population-based case-control study was 3%. Thus, 25, 15, and 400 individuals with diabetes in the CCHS, the CGPS, and at the Steno Diabetes Center could have been avoided if they had not had elevated transferrin saturation.

The corresponding population attributable risks for Type 1 diabetes were 4% for the CCHS, 1% for the CGPS, 22% for the population-based case-control study, and 3% combined.

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Likewise, the corresponding population attributable risks for Type 2 diabetes were 3% for the CCHS, 1% for the CGPS, 1% for the population-based case-control study, and 1% combined.

Hemochromatosis genotypes among individuals with elevated transferrin saturation

The genotype distribution was not statistically different (p=0.08) in individuals with diabetes in the general population studies versus Steno Diabetes Center or in individuals with diabetes vs. no diabetes in the general population studies only (Online-only Table 2); thus the risk of increased transferrin saturation observed in our study is not secondary to an increased risk conferred by the genotype.

Conclusions

The risk of increased transferrin saturation observed in our study was not secondary to an increased risk conferred by the C282Y or H63D genotype. **Other genetic factors (not investigated in this study) that are major determinants of serum iron and transferrin saturation in the general population, could potentially also influence our results(21,22), and an increased risk of transferrin saturation could theoretically also be secondary to diabetes.**

Diabetes as a consequence of hemochromatosis is characterised by both insulin resistance and beta-cell destruction(23), and the metabolic phenotype can therefore mimic both Type 2 diabetes and non-autoimmune Type 1 diabetes. The defects in both insulin-producing and insulin-sensitive tissues are most likely caused by iron-dependent catalysis via the Fenton reaction of reactive oxygen radical species, which impair insulin signalling in skeletal muscle and liver and cause beta-cell destruction due to insufficient beta-cell deficient antioxidant defence(24), with main emphasis on beta-cell destruction in Type 1 diabetes and insulin resistance in Type 2 diabetes(25).

Strengths of our work include the fact that the patients with diabetes was a large group, representative, and well defined, thereby reducing the possibility of bias. The patients in the case-control study almost exclusively came from Copenhagen County, and therefore formed an unselected representative group. Misclassification of the subjects in the case-control study is unlikely, since general practitioners diagnosed the patients and then referred them to Steno Diabetes Center, a highly sub-specialised diabetes-clinic. The strength of the general population studies was that they were large and subjects sampled randomly.

Limitations in our study were that the sub-classification of diabetes was not as well-defined in the general population studies as in the case-control study and that we studied Caucasians only and therefore our results may not apply to other races. **Also, the meta-analytical approach to include the three study populations to maximize statistical power is open to selection bias.**

We cannot exclude that some individuals with high transferrin saturation could have a bone marrow disorder with reduced or high iron turnover such as hypoplastic anemia or haemolytic disease, hepatocellular injury, or a transient non-specific rise in transferrin saturation rather than increased iron stores; however, a significant contribution such of conditions is unlikely because of the rarity of these diseases.

In context of existing literature three other studies(8,9,11) have used transferrin saturation as the primary independent variable (without considering hemochromatosis genotype) for the study of the risk of diabetes. However, these studies are heterogeneous concerning ethnicity (two of the studies use mixed populations(8,9), another doesn't report ethnicity(11)), study design (cross-sectional(9), retrospective cohort(8), and case-study with historical controls(11)), size (44000 Whites/53000 other ethnicities(9), 9000(8), and 820(11)), transferrin saturation threshold (no threshold but only mean values(9), threshold of at least 45%(8), threshold of 35%(11)), diabetes diagnosis (any diabetes(8,9), Type 1(11), Type 2(11)), source of diabetes diagnosis (self-report(8,9), use of ICD-9 code(8) for diabetes patients in an endocrinology department(11)), and finally also results (one in favour(11) and

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two not infavour(8,9) of association of elevated transferrin saturation and risk of diabetes). In comparison, our study consisted of three individual studies consistent in study design (two large general population studies ascertained alike, and a case-control study using patients with diabetes ascertained from a similar population as the general population studies and compared to controls ascertained like the population studies with no overlap of individuals in the three individual studies), ethnicity (only White individuals), transferrin saturation threshold value of 50% in accordance with accepted clinical practice(26-28), diabetes diagnosis (any diabetes, Type 1 and Type 2 for all three studies), source of the diabetes diagnosis (self-report, medication, ICD-coding, and a non-fasting glucose above 11 mmol/L), and finally also consistent in results.

In conclusion, transferrin saturation ≥50% was associated with 2-3 fold increased risk of any diabetes mellitus, Type 1 diabetes, and Type 2 diabetes, independently of hemochromatosis genotype.

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SUPPLEMENTARY DATA

Supplementary Table 1. Characteristics of participants.

	CCHS	CGPS	Population-based case-control study
Total participants, N	9121	24195	12258
Recruitment date	1991-1994	2003-2007	2001-2007
Diabetes mellitus, N	910[1]	1496[2]	6129[3]
Type 1, N*	274	393	2664
Type 2, N**	875	1418	3465
Participants without diabetes	8211	22699	6129
Age, years	60(48 to 70)	59(49 to 68)	56(42 to 66)
Females, %	57	54§	44
Transferrin saturation, %	27(21 to 34)	22(17 to 28)§	24(19 to 31)
Transferrin >=50%, N	355	346	361
BMI>=25, %	52	57§	_
Hemoglobin concentration, mM	8.7(8.2 to 9.2)	8.8(8.3 to 9.3)	_

Age, transferrin saturation, and Hemoglobin concentration are median with interquartile ranges.
CCHS: Copenhagen City Heart Study.
CGPS: Copenhagen General Population Study.
Population-based case-control study: cases from Steno Diabetes Center matched 1:1 on age and gender with controls ascertained like in the CGPS.
There is no overlap of individuals between the three studies.
*Those receiving insulin, or having ICD codes (ICD8: 249, ICD10:E10).
**Those receiving other diabetic medication or having ICD codes (ICD8:250, ICD10: E11, E13, E14).
[1]: 239 patients has ICD codes for both Type 1 and Type 2 diabetes.
[2]: 315 patients has ICD codes for both Type 1 and Type 2 diabetes.
[3]: The Type 1 and Type 2 diagnoses are exclusive for the patients collected at Steno Diabetes Center.
§Number of females, number of individuals with BMI>=25, and transferrin saturation differed significantly between CCHS and CGPS ($p<0.001$).

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SUPPLEMENTARY DATA

Supplementary Table 2. Hemochromatosis genotype among individuals with transferrin saturation≥50%.

	General population studies*: Diabetes N(%)	General population studies*: No diabetes N(%)	Steno Diabetes Centre**: Diabetes N(%)
Wild type/wild type	37(44)	207(35)	131(52)
H63D/wild type	13(15)	129(22)	57(23)
H63D/H63D	3(4)	39(7)	8(3)
C282Y/wild type	14(17)	90(15)	33(13)
C282Y/H63D	11(13)	64(10)	13(5)
C282Y/C282Y	6(7)	67(11)	10(4)
Total	84 (100)	596(100)	252(100)

General population studies: CCHS (Copenhagen City Heart Study) and CGPS (Copenhagen General Population Study).
Steno: Steno Diabetes Centre, Copenhagen.
*4 did not have any genotype.
**22 did not have any genotype.
Transferrin saturation among those lacking genotype was not different from those having a genotype.
P-value for the distribution of hemochromatosis genotypes among individuals with diabetes mellitus versus without diabetes mellitus in the general population studies: p=0.3.
P-value for the distribution of hemochromatosis genotypes among individuals with diabetes mellitus in the general population studies versus Steno Diabetes Centre: p=0.08.

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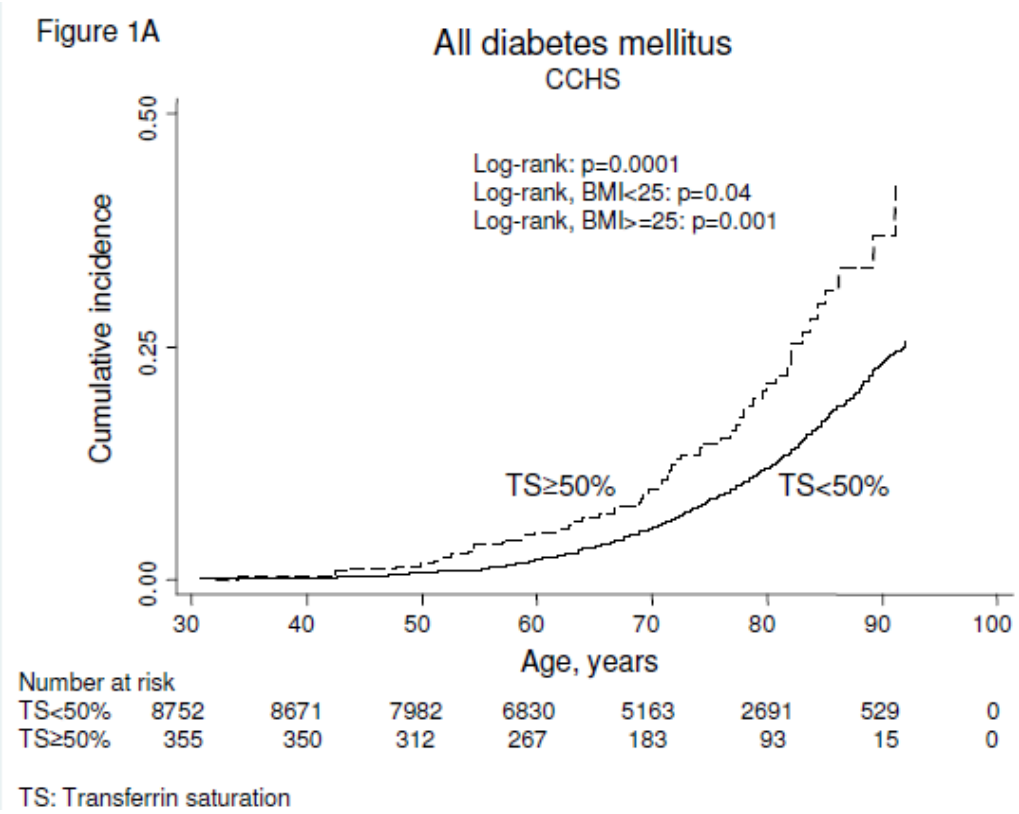
SUPPLEMENTARY DATA

Supplementary Figure 1. Cumulative incidence by age of any diabetes mellitus according to transferrin saturation in two general population studies. CCHS, Copenhagen City Heart Study (1A). CGPS, Copenhagen General Population Study (1B). Based on Kaplan-Meyer estimates. **BMI: Body mass index.**



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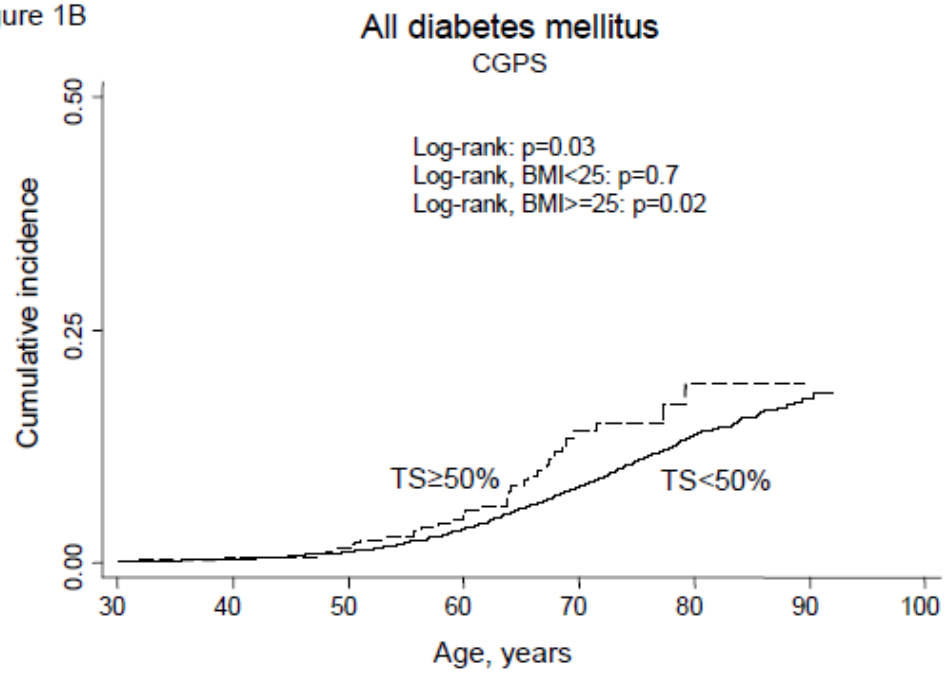
SUPPLEMENTARY DATA

Figure 1B



Number at risk

TS<50%	23616	22950	19622	13667	6869	2093	156	0
TS≥50%	343	336	294	207	108	24	0	0

TS: Transferrin saturation

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