Diabetologia (2013) 56:1623–1628
DOI 10.1007/s00125-013-2897-x

SHORT COMMUNICATION

Expression of anti-inflammatory macrophage genes within skeletal muscle correlates with insulin sensitivity in human obesity and type 2 diabetes

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Received: 21 December 2012 / Accepted: 6 March 2013 / Published online: 18 April 2013
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Abstract
Aims/hypothesis Low-grade systemic inflammation and adipose tissue inflammatory macrophages are frequently detected in patients with obesity and type 2 diabetes. Whether inflammatory macrophages also increase in skeletal muscle of individuals with metabolic disorders remains controversial. Here, we assess whether macrophage polarisation markers in skeletal muscle of humans correlate with insulin sensitivity in obesity and type 2 diabetes.
Methods Skeletal muscle biopsies were obtained from individuals of normal weight and with normal glucose tolerance (NGT), and overweight/obese individuals with or without type 2 diabetes. Insulin sensitivity was determined by euglycaemic–hyperinsulinaemic clamps. Expression of macrophage genes was analysed by quantitative RT-PCR.
Results Gene expression of the inflammatory macrophage phenotype marker cluster of differentiation (CD)11c was higher in muscle of type 2 diabetes patients (p=0.0069), and correlated with HbA_{1c} (p=0.0139, ρ=0.48) and fasting plasma glucose (p=0.0284, ρ=0.43), but not after correction for age. Expression of *TGFB1*, encoding the anti-inflammatory marker TGF-β1, correlated inversely with HbA_{1c} (p=0.0095, ρ=−0.50; p=0.0484, ρ=−0.50) and fasting plasma glucose (p=0.0471, ρ=−0.39; p=0.0374, ρ=−0.52) in two cohorts, as did HbA_{1c} with gene expression of macrophage galactose-binding lectin (MGL) (p=0.0425, ρ=−0.51). *TGFB1* expression was higher in NGT individuals than in individuals with type 2 diabetes (p=0.0303), and correlated with low fasting plasma insulin (p=0.0310, ρ=−0.42). In exercised overweight/obese individuals, expression of genes for three anti-inflammatory macrophage markers, MGL (p=0.0031, ρ=0.71), CD163 (p=0.0268, ρ=0.57) and mannose receptor (p=0.0125, ρ=0.63), correlated with high glucose-disposal rate.
Conclusions/interpretation Muscle expression of macrophage genes reveals a link between inflammatory macrophage markers, age and high glycaemia, whereas anti-inflammatory markers correlate with low glycaemia and high glucose-disposal rate.

Keywords Exercise · Inflammation · Insulin resistance · Mannose receptor · Macrophage galactose-binding lectin · Macrophage polarisation · Muscle insulin action · Type 2 diabetes

Abbreviations

CD	Cluster of differentiation
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
MGL	Macrophage galactose-binding lectin
MR	Mannose receptor
NGT	Normal glucose tolerance

Introduction

In humans, obesity increases the risk of developing type 2 diabetes. Inflammatory cells, particularly macrophages in

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visceral adipose tissue, have been implicated in the progression of obesity to type 2 diabetes [1]. Skeletal muscle is the primary site of dietary glucose disposal in humans, and muscle insulin resistance is essential for whole-body insulin resistance [2]. Numerous studies support the concept that skeletal muscle cells (fibres) develop insulin resistance as a result of the lipotoxic environment on high fat-feeding and obesity [3]. In vitro, muscle cells exposed to saturated fatty acids develop insulin resistance and mount a cell-autonomous inflammatory response [4]. On the other hand, there is evidence that, in vivo, muscle insulin resistance is reactive to factors emanating from adipose and hepatic tissues, in particular adipose tissue macrophage infiltration [4].

However, a third possible scenario remains untested, namely that macrophages with inflammatory signatures increase within skeletal muscle beds and contribute locally to muscle insulin resistance. Muscle-infiltrating macrophages in obesity might affect muscle glucose metabolism via secreted factors that impair glucose homeostasis. Although the rise in human muscle inflammatory genes in obesity and type 2 diabetes correlates with insulin resistance [5], evidence for elevated immune cells in human muscle in obesity and type 2 diabetes is scant and controversial [6, 7]. Moreover, the phenotypic polarisation of muscle macrophages in obesity and type 2 diabetes is unknown, as is any possible correlation to metabolic status. Here, we assess the expression of genes reflecting different macrophage phenotypes in human muscle biopsies and establish correlations with whole-body insulin action.

Methods

Study participants and muscle biopsies All studies were approved by the Ethics Committee of the University of Leipzig, Germany, and all participants gave written informed consent. Individuals in cohorts I and II were divided into those with normal glucose tolerance (NGT) and those with type 2 diabetes on the basis of a 75 g oral glucose tolerance test according to American Diabetes Association criteria: participants with NGT had fasting plasma glucose (FPG) <6.0 mmol/l and 120 min plasma glucose <7.8 mmol/l; those with type 2 diabetes had FPG ≥6.0 mmol/l and/or 120 min plasma glucose >11.1 mmol/l.

Cohort III comprised individuals with or without previous type 2 diabetes diagnosis and metformin requirement, who underwent a documented 12 month exercise programme: 60 min aerobic training twice weekly (20 min warming and cool down, 20 min running or cycling and 20 min resistance training) plus 60 min swimming once weekly. Insulin sensitivity and glucose disposal were assessed by euglycaemic–hyperinsulinaemic clamps. Skeletal muscle and blood samples were collected 8–10 h after an overnight fast. Plasma was analysed for glucose, insulin, HbA_{1c} and C-reactive protein. Vastus lateralis muscle biopsies (<100 mg) were obtained under local anaesthesia and immediately snap frozen in liquid nitrogen.

Gene expression analysis Frozen muscle tissue was homogenised in Trizol, RNA isolated (RNeasy Mini kit, Qiagen, Mississauga, ON, Canada) and cDNA generated (SuperScript VILO cDNA Synthesis Kit, Life Technologies, Burlington, ON, Canada). Quantitative PCR was performed with Gene Expression master mix (Life Technologies) using a 7900HT Fast Real-Time PCR System (Life Technologies). Specific gene expression was measured with the following Taqman assays in all samples (Life Technologies): *ITGAM*, encoding cluster of differentiation (CD)11b (Hs00355885_m1); *ITGAX*, encoding CD11c (Hs01015064_m1); *CD163* (Hs00174705_m1); *CLEC10A*, encoding macrophage galactose-binding lectin (MGL) (Hs00197107_m1); *MRC1*, encoding mannose receptor (MR) (Hs00267207_m1); *TNF* encoding TNF-α (Hs00174128_m1); and *TGFB1*, encoding TGF-β1 (Hs00998133_m1). The cDNA input was 8 ng/reaction. Comparative cycle threshold results were expressed relative to the reference gene Ras-like without CAAX 1 (*RIT1*, Hs00608424_m1).

Gene expression data were analysed by Kruskal–Wallis test and Spearman rank correlation analysis. Pre- and post-exercise data for BMI and glucose-disposal rate (R_d) were compared by Wilcoxon matched-pairs signed-rank test. Values of $p<0.05$ were considered significant. Only results with significant p values are shown.

Results and Discussion

ITGAX expression in muscle correlates positively with glycaemia In cohort I (Table 1), we tested the hypothesis that markers of pro- and anti-inflammatory macrophages in muscle biopsies correlate with metabolic variables in lean and obese NGT individuals, and in type 2 diabetes patients. The expression of the pro-inflammatory macrophage gene *ITGAX* (encoding CD11c) correlated with FPG (Fig. 1a, $p=0.0284$) and HbA_{1c} (Fig. 1b, $p=0.0139$). *ITGAX* was not expressed by cultured human myotubes (data not shown). *ITGAX* expression tended to correlate inversely with R_d (not shown, $p=0.0568$) and also to increase with age (not shown, $p=0.0552$). When adjusted for age, *ITGAX* expression no longer correlated significantly with HbA_{1c} (Fig. 1c, $p=0.0933$) or FPG (not shown, $p=0.1819$). Expression of the anti-inflammatory cytokine *TGFB1* (primarily expressed by leucocytes, including macrophages) correlated negatively with FPG (Fig. 1a, $p=0.0095$), HbA_{1c} (Fig. 1b, $p=0.0471$) and fasting plasma insulin (FPI) (Fig. 1d, $p=0.0310$).

Table 1 Patient characteristics per cohort

Cohort	Lean, NGT	Overweight/obesity, NGT	Overweight/obesity, T2D
I			
Number	8	8	10
Age (years)	37.1 (28–45)	41.0 (32–51)	49.8 (43–55)*†
Sex (% male)	63	38	30
BMI (kg/m^2)	24.4 (23.6–25.0)	37.1 (30.6–45.8)*	33.9 (27.8–42.6)*
Body fat (%)	19.8 (16.8–22.6)	33.7 (23.1–43.9)*	31.6 (25.4–40.5)*
HbA_{1c} (%)	5.3 (5.1–5.6)	5.7 (5.5–5.9)	6.2 (5.8–6.7)*†
HbA_{1c} (mmol/mol)	34 (32–38)	39 (37–41)	44 (40–50)*†
R_d (μmol kg^{-1} min^{-1})	100.6 (77–116)	69.1 (43–94)	41.7 (15–64)*
FPG (mmol/l)	5.3 (4.9–5.5)	5.8 (5.4–6.1)	6.4 (5.8–7.2)*
FPI (pmol/l)	8.7 (1.9–24.3)	126.5 (55.9–208.4)*	169.6 (97.5–268)*
C-reactive protein (nmol/l)	6.7 (1.9–12.4)	21.0 (8.6–33.3)*	22.9 (11.4–35.2)*
II			
Number	5	5	6
Age (years)	46.2 (45–48)	46.4 (45–48)	47.0 (45–48)
Sex (% male)	40	40	67
BMI (kg/m^2)	23.4 (21.5–24.5)	30.7 (26.0–32.6)	33.3 (32.8–34.3)*
Body fat (%)	30.2 (24.5–37.4)	33.8 (30.3–35.1)	34.4 (32.7–38.2)
HbA_{1c} (%)	5.4 (5.2–5.6)	5.5 (5.4–5.6)	6.1 (5.8–6.4)*
HbA_{1c} (mmol/mol)	36 (33–38)	37 (36–38)	43 (40–46)*
R_d (μmol kg^{-1} min^{-1})	112.6 (108–117)	96.0 (86–105)	44.2 (37–56)*
FPG (mmol/l)	5.0 (4.5–5.3)	5.2 (4.7–5.4)	6.2 (5.5–7.1)*
FPI (pmol/l)	9.4 (6.3–12.5)	15.7 (9.7–25.4)	94.1 (76.3–109.2)*
C-reactive protein (nmol/l)	3.8 (2.9–5.7)	21.9 (8.6–45.7)	27.6 (17.1–39.0)*
III, post-exercise			
Number	–	9	6
Age (years)	–	47.4 (36–53)	50.3 (47–55)
Sex (% male)	–	78	67
BMI (kg/m^2)	–	33.5 (25.3–42.3)	30.7 (25.1–36.5)
Body fat (%)	–	30.3 (19.8–40.3)	27.7 (22.6–31.7)
HbA_{1c} (%)	–	6.0 (5.6–6.4)	6.3 (5.9–6.5)†
HbA_{1c} (mmol/mol)	–	42 (38–45)	45 (41–48)†
R_d (μmol kg^{-1} min^{-1})	–	68.0 (46–88)	66.8 (55–99)
FPG (mmol/l)	–	6.2 (5.4–8.1)	6.3 (5.8–6.8)
FPI (pmol/l)	–	144.6 (62.7–274.0)	92.7 (2.9–150.4)
C-reactive protein (nmol/l)	–	16.0 (7.6–29.5)	18.9 (13.3–22.9)
Metformin (% recipients)	–	33	67

Data shown are means and ranges

All individuals with type 2 diabetes in cohorts I and II were newly diagnosed and did not receive diabetes medication; seven individuals in cohort III received metformin during the exercise programme

Significant differences between values are indicated (Mann–Whitney test for two groups, Kruskal–Wallis test for three groups) *$p<0.05$ vs lean, NGT; †$p<0.05$ vs overweight/obesity, NGT

T2D, type 2 diabetes

The association of *ITGAX* with metabolic disease was substantiated by elevated *ITGAX* expression in ten patients with type 2 diabetes and BMI >25 kg/m^2 compared with eight individuals with NGT and BMI >30 kg/m^2 (Fig. 1e, $p=0.0069$). However, this difference was not significant after correcting for age (Fig. 1f, $p=0.0542$). Expression of *ITGAM* , encoding the pan-macrophage marker CD11b, was also higher in the type 2 diabetes group (Fig. 1e, $p=0.0094$) and did not correlate with age.

Hence, we demonstrate that expression of the pro-inflammatory macrophage 'M1' marker CD11c correlates positively with the metabolic disturbances in type 2 diabetes. However, the increase in muscle *ITGAX* expression in type 2 diabetes may be an age-dependent phenomenon (see below).

TGFB1 and MGL expression in muscle correlates negatively with glycaemia A second, age-matched cohort of individuals was recruited to re-test the hypothesis (cohort II, Table 1). Importantly, *ITGAX* expression did not differ between NGT and type 2 diabetes; however, similar to cohort I, *TGFB1* expression was lower with higher FPG (Fig. 1g, $p=0.0374$)

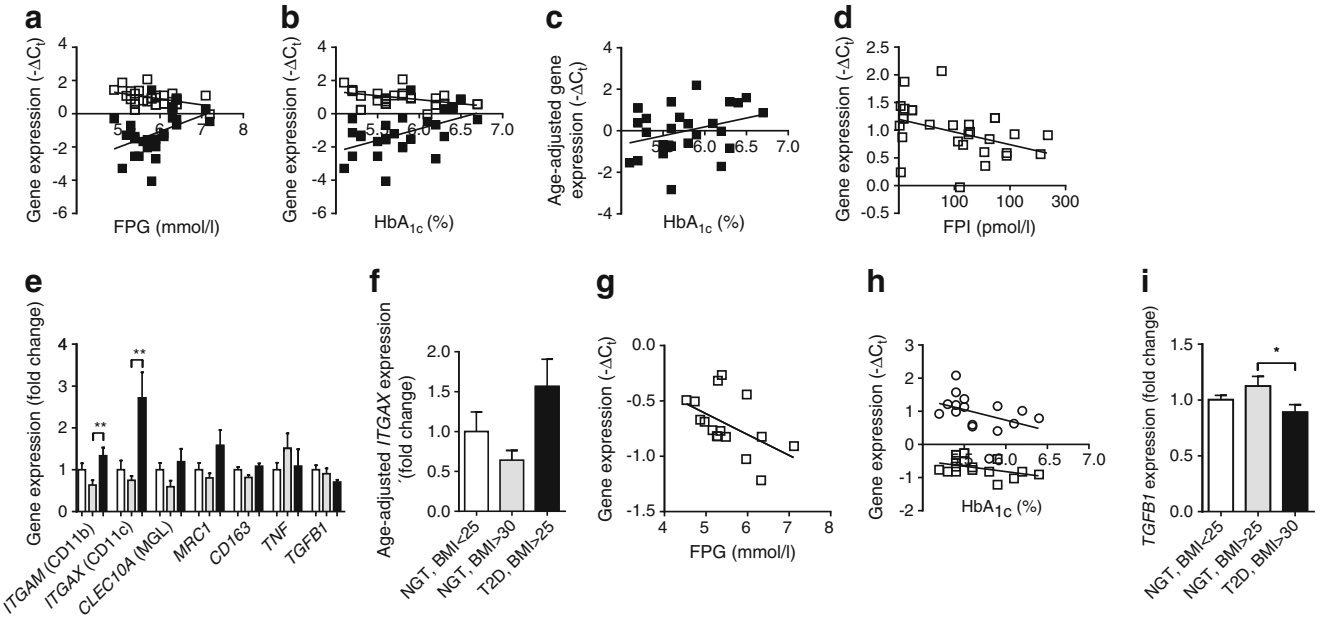

Fig. 1 (**a**–**d**) Correlations between vastus lateralis muscle gene expression and metabolic variables in individuals from cohort I (n=26). (**a**) Expression of *ITGAX*, encoding CD11c (black squares; p=0.0284; ρ=0.43) correlates positively with FPG, and *TGFB1* expression (white squares; p=0.0471; ρ=−0.39) correlates negatively with FPG by Spearman rank correlation analysis. (**b**) *ITGAX* expression (black squares; p=0.0139; ρ=0.48) correlates positively and *TGFB1* expression (white squares; p=0.0095; ρ=−0.50) correlates negatively with HbA_{1c}. (**c**) After correction for age, expression of *ITGAX* (black squares) tends to correlate with HbA_{1c} (p=0.0933; ρ=0.34). (**d**) Expression of *TGFB1* correlates negatively with FPI (white squares; p=0.0310; ρ=−0.42). To convert values for HbA_{1c} in % into mmol/mol, subtract 2.15 and multiply by 10.929. (**e**) Relative gene expression in muscle in groups of individuals from cohort I (mean±SEM; white bars, NGT; BMI <25 kg/m^2; grey bars, NGT, BMI >30 kg/m^2; black bars, type 2 diabetes, BMI >25 kg/m^2). Expression of *ITGAM* (p=0.0094) and *ITGAX* (p=0.0069) differed significantly between obese NGT patients and overweight/obese type 2 diabetes patients (Kruskal–Wallis test). (**f**) *ITGAX* expression tends to differ between obese NGT and type 2 diabetes patients, when corrected for age (p=0.0542). (**g**–**h**) Correlations between muscle gene expression and metabolic variables in cohort II (n=16). (**g**) *TGFB1* (white squares; p=0.0374; ρ=−0.52) correlates negatively with FPG. (**h**) Expression of *TGFB1* (white squares; p=0.0484; ρ=−0.50) and *CLEC10A* (white circles; p=0.0425; ρ=−0.51) correlates negatively with HbA_{1c}. (**i**) Type 2 diabetes patients have lower *TGFB1* expression in muscle compared with overweight/obese NGT individuals in cohort II (mean±SEM shown; p=0.0303). Expression of all genes was normalised to the expression of *RIT1* for each sample: $\Delta C_t = C_{t(\text{test gene})} - C_{t(\text{reference gene})}$. Comparative gene expression was calculated as $\Delta\Delta C_t = \Delta C_{t(\text{disease group})} - \Delta Ct_{(\text{control group})}$. Fold-change values were calculated as $2^{-\Delta\Delta C_t}$. Correlations were based on $-\Delta C_t$ values, as a low ΔC_t value indicates high expression. BMI is given in kg/m^2. $^*p$<0.05; $^{**}p$<0.01. T2D, type 2 diabetes

and HbA_{1c} (Fig. 1h, p=0.0484), and tended to correlate positively with R_d (not shown, p=0.0609).

To detect 'M2'-polarised (i.e. non-inflammatory) macrophage markers, we analysed the marker genes *CLEC10A* (MGL), *MRC1* (MR) and *CD163*. *CLEC10A* expression was inversely correlated with HbA_{1c} (Fig. 1h, p=0.0425) and tended to correlate negatively with FPG (not shown, p=0.0639). *MRC1* and *CD163* expression did not correlate significantly with metabolic variables. *TGFB1* expression was significantly reduced in type 2 diabetes patients

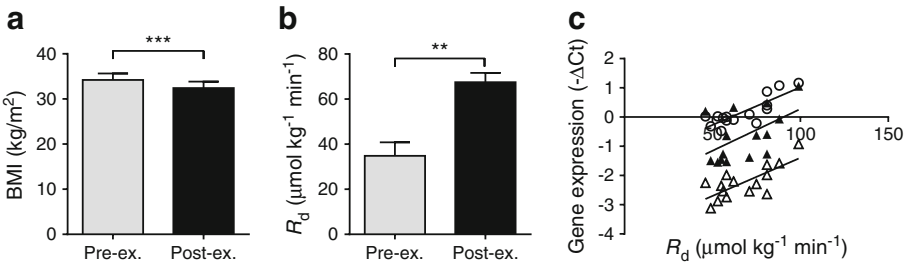

Fig. 2 (**a**, **b**) Significant effects of a 1 year exercise intervention programme on (**a**) BMI (p=0.0002) and (**b**) glucose-disposal rate (p=0.0010) in individuals with a BMI >25 kg/m^2 (cohort III; n=15; mean±SEM; Wilcoxon matched-pairs signed-rank test). (**c**) Correlations between muscle gene expression of anti-inflammatory macrophage genes and insulin sensitivity in cohort III. Expression of *CLEC10A* (white circles; p=0.0031; ρ=0.71), *CD163* (black triangles; p=0.0268; ρ=0.57) and *MRC1* (white triangles; p=0.0125; ρ=0.63) correlates with glucose-disposal rate during clamping (R_d). $^{**}p$<0.01; $^{***}p$<0.001. Post-ex., after exercise; pre-ex., before exercise

compared with overweight/obese NGT individuals (Fig. 1i). In this cohort, individuals with NGT and type 2 diabetes differed markedly in FPI levels. Thus, high *TGFB1* expression clusters with low FPI.

Taken together, *TGFB1* expression differed between NGT and type 2 diabetes patients in one cohort and correlated with low HbA_{1c} and FPG in two cohorts, independent of age. This raises the interesting possibility that muscle TGF-β1, although implicated in adipose tissue remodelling and fibrosis in obesity [8], may also influence blood glucose homeostasis.

MGL, MRC1 and CD163 expression correlates with insulin sensitivity in trained overweight humans Finally, muscle biopsies were obtained from 15 individuals with BMI >25 kg/m^2 after 1 year of prescribed physical activity (cohort III, Table 1). Exercise had a pronounced beneficial effect on BMI (Fig. 2a, p=0.0002 for pre-exercise vs post-exercise) and, particularly, R_d (Fig. 2b, p=0.0010). After the exercise regimen, R_d in both NGT and type 2 diabetes groups (68.0 and 66.8 μmol kg^{-1} min^{-1}, respectively) was higher than R_d in type 2 diabetes in both other cohorts (41.7–44.2 μmol kg^{-1} min^{-1} range) (Table 1). Expression of the anti-inflammatory markers *CLEC10A* (p=0.0031), *MRC1* (p=0.0125) and *CD163* (p=0.0268) correlated strongly with R_d (Fig. 2c). There were no significant differences in gene expression between individuals with NGT and type 2 diabetes at this endpoint. Hence, expression of the anti-inflammatory 'M2' markers *MRC1*, *CLEC10A* and *CD163* correlated with better insulin sensitivity in this group of overweight/obese people. This might suggest that the phenotype of muscle-resident macrophages and/or of a recruited population may be impacted by exercise intervention. Further studies are required to explore these possibilities.

Exercise and weight loss have marked beneficial effects in lowering systemic inflammation markers in the plasma of humans; however, this is not necessarily reflected in the macrophage content within skeletal muscle [6, 9]. Muscle levels of CD68 did not change with a 15 week weight-loss programme in 27 people with a mean initial BMI of 45.8 kg/m^2 [9]. Similarly, no differences in net macrophage number or gene expression were observed before and after exercise in patients with type 2 diabetes and BMI ≥30 kg/m^2 [6]. Since another study failed to detect weight-loss-dependent alterations in adipose tissue [10], weight loss may not act oppositely to weight gain or greater weight loss may be necessary to modify macrophage numbers. Although we did not have pre-exercise samples, we found that the anti-inflammatory 'M2' macrophage markers *CLEC10A*, *MRC1* and *CD163* strongly correlated with muscle insulin sensitivity in exercised people with BMI >25 kg/m^2.

Conclusion

In summary, we show that pro- and anti-inflammatory macrophage marker genes vary inversely in human muscle, depending on age, glycaemic status and insulin sensitivity. As muscle insulin sensitivity can be affected by some of the same factors as adipocyte insulin action, it is conceivable that the phenotype of the muscle-residing macrophages affects muscle insulin sensitivity. We further show that low expression of *CLEC10A* and *TGFB1* is associated with hyperglycaemia and that low *CLEC10A* is also associated with low glucose-disposal rate. Optimal polarisation of macrophages may contribute to the amelioration of insulin resistance with routine exercise.

Acknowledgements The technical assistance of Z. Liu is highly appreciated, and we thank P. Bilan for knowledgeable advice on the study and manuscript (both Cell Biology Program, The Hospital for Sick Children, Toronto, Canada).

Funding Patient analysis and sample collection was supported by Deutsche Forschungsgemeinschaft, Clinical Research group 'Atherobesity' (KFO152; BL 833/1-1). Biopsy analysis was carried out at The Hospital for Sick Children, supported by Canadian Institutes of Health Research (CIHR) grant 12601 to A. Klip. L. N. Fink was supported by fellowships from the Carlsberg Foundation and Novo Nordisk. S. R. Costford was supported by a Mitacs fellowship.

Contribution statement LNF, AS and AK designed the study. LNF, SRC and KLC acquired and analysed gene expression data. AO and MB recruited patients and controls, collected muscle biopsies and analysed metabolic variables. LNF and AK wrote the manuscript. SRC, KLC, AO, MB and AS revised the manuscript. All authors approved the final version.

Duality of interest L. N. Fink and A. Sams are employed by Novo Nordisk. All other authors declare that there is no duality of interest associated with their contribution to this manuscript

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