ARTICLE

Clinical Research

Evaluation of chitotriosidase as a biomarker for adipose tissue inflammation in overweight individuals and type 2 diabetic patients

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Abstract

Background Overweight and obesity can lead to adipose tissue inflammation, which causes insulin resistance and on the long-term type 2 diabetes mellitus (T2D). The inflammatory changes of obese-adipose tissue are characterized by macrophage infiltration and activation, but validated circulating biomarkers for adipose tissue inflammation for clinical use are still lacking. One of the most secreted enzymes by activated macrophages is chitotriosidase (CHIT1).

Objective To test whether circulating CHIT1 enzymatic activity levels reflect adipose tissue inflammation.

Methods Plasma and adipose tissue samples of 105 subjects (35 lean, 37 overweight, and 33 T2D patients) were investigated. CHIT1 mRNA levels were determined in adipose tissue-resident innate immune cells. CHIT1 mRNA levels, protein abundance, and plasma enzymatic activity were subsequently measured in adipose tissue biopsies and plasma of control subjects with varying levels of obesity and adipose tissue inflammation as well as in T2D patients.

Results In adipose tissue, CHIT1 mRNA levels were higher in stromal vascular cells compared to adipocytes, and higher in adipose tissue-residing macrophages compared to circulating monocytes ($p < 0.001$). CHIT1 mRNA levels in adipose tissue were enhanced in overweightcompared to lean subjects and even more in T2D patients ($p < 0.05$). In contrast, plasma CHIT1 enzymatic activity did not differ between lean, overweight subjects and T2D patients. A mutation of the *CHIT1* gene decreases plasma CHIT1 activity.

Conclusions CHIT1 is expressed by adipose tissue macrophages and expression is higher in overweight subjects and T2D patients, indicating its potential as tissue biomarker for adipose tissue inflammation. However, these differences do not translate into different plasma CHIT1 activity levels. Moreover, a common *CHIT1* gene mutation causing loss of plasma CHIT1 activity interferes with its use as a biomarker of adipose tissue inflammation. These results indicate that plasma CHIT1 activity is of limited value as a circulating biomarker for adipose tissue inflammation in human subjects.

Introduction

Individuals that are overweight or obese have a high risk to develop metabolic diseases such as type 2 diabetes (T2D) [1–3]. During the last decades, an increased number of studies suggest that obesity-induced adipose tissue inflammation is an important pathogenic mediator of the development of insulin resistance and the subsequent T2D

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development [4–6]. Adipose tissue enlarges often due to excessive caloric intake, which results in adipocyte hypertrophy leading to an impaired metabolic function [1, 3]. Macrophages are key players in the onset of adipose tissue inflammation [7] with increased infiltration into the adipose tissue as a morphological hallmark of obese-adipose tissue inflammation [8–11]. Therefore, levels of macrophage activity in adipose tissue reflect the inflammatory state of adipose tissue and predict the risk to develop related metabolic complications. Hence, biomarkers for adipose tissue inflammation will likely be of high clinical diagnostic value to identify (obese) individuals at risk for type 2 diabetes or related metabolic complications. The enzyme chitotriosidase (CHIT1) is one of the most abundant secreted protein by activated macrophages [12, 13] and could, therefore, be considered as a potential biomarker to determine the inflammatory condition of adipose tissue. Initially being discovered and described as a valuable biomarker for Gaucher disease, other findings describe CHIT1 as an important regulator in immunological processes that play a relevant role in several diseases [12–14]. Its most prominent physiological role is to hydrolyze the β-[1, 4]-linkage of chitin, a major component of cell membranes of fungi and human pathogens [15].

Increased plasma CHIT1 activity has been reported in obese individuals with impaired glucose tolerance and in T2D patients compared to controls [16–18]. In addition, plasma CHIT1 activity was found to be positively associated with other important predecessors and complications of T2D (i.e., glycemic control, atherosclerosis and kidney dysfunction) [13, 17, 19]. However, whether the abundance and activity of CHIT1 reflects obesity-induced adipose tissue inflammation is yet unknown. The current study therefore aims to evaluate whether CHIT1 is expressed in adipose tissue and relates to adipose tissue inflammation, in the context of obesity and T2D. We hypothesized that CHIT1 is higher expressed in overweight and T2D subjects and correlates with adipose tissue inflammation. To investigate this, we determined CHIT1 levels in adipose tissue and plasma of individuals with a varying range of body mass index (BMI), ranging from lean to obese, as well as in patients with type 2 diabetes combined with detailed characterization of adipose tissue inflammation.

Materials and methods

Subjects

One hundred and five participants were included in this study, consisting of control participants ($n = 72$), with a broad BMI range (20.1–39.8 kg/m^2), and T2D patients ($n = 33$). The control participants were subdivided into a lean and overweight control group, whereas the T2D patients were BMI matched to the overweight control group and were on long-term stable oral glucose-lowering treatment according to national guidelines, without insulin administration. Subject characteristics of control participants [20, 21] and T2D patients [22] have been described previously [23]. Exclusion criteria for T2D patients were other types of diabetes, significant cardiovascular, renal, liver or other co-morbidity, use of corticosteroids, uncontrolled endocrine disorders (stable supplementation with thyroid hormone was allowed), bariatric treatment, excessive alcohol consumption (>20 g/day), drug abuse, and use of thiazolidinedione derivatives. From all individuals, blood was taken after an overnight fast and anthropometric tests were performed. Body composition was assessed by using formulas described by Jackson and Pollock [24]. In addition, subcutaneous adipose tissue biopsies were obtained. Study protocols were approved by the local ethical committee. All study participants provided written consent.

Biochemical analysis

Plasma glucose concentrations and HbA1c were measured by standard laboratory methods. Furthermore, plasma IL-8 and high-sensitive C-reactive protein (hsCRP) were measured by ELISA (R&D systems, Minneapolis, MN, USA).

Adipose tissue inflammation

Subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies 6–10 cm lateral to the umbilicus. Immunohistochemistry to detect macrophages was performed using a CD68-monoclonal antibody (AbD Serotec, Oxford, UK). The percentage of macrophages was expressed as the total number of CD68-positive cells divided by the total number of adipocytes counted in 20 random microscopic fields. A crown-like structure was defined as an adipocyte surrounded by at least three macrophages [25].

Protein levels of interleukin (IL)−6, IL-8 and monocyte chemoattractant protein (MCP-1) within the adipose tissue were measured in adipose tissue lysates by Luminex fluorescent bead human cytokine immunoassays (Milliplex Map, Millipore Corp, Billerica, MA), as described previously [22].

Mature adipocytes and stromal vascular fraction

Paired subcutaneous (SAT) and visceral adipose tissue (VAT) were obtained from 7 subjects, during cholecystectomy (subject characteristics are depicted in supplementary table 1). Samples were disaggregated and digested using collagenase type 1 (Gibco, Life Technologies) to isolate mature adipocytes (MA) and the stromal vascular fraction (SVF), as described before [21]. The cellular

fractions were subsequently used for RNA isolation, followed by real-time PCR analysis, as described below.

Isolation of CD14+ cells

Blood, visceral and subcutaneous adipose tissue were obtained from patients undergoing surgery (subject characteristics are depicted in supplementary table 1). Informed consent was obtained before the surgical procedure and the study protocol was approved by the ethical committee of the Radboud university medical center. CD14+ cells were isolated from blood using MACS selection. CD14+ cells from both subcutaneous and visceral adipose tissue were isolated from the stromal vascular fraction using MACS selection. The stromal vascular fraction was obtained by collagenase digestion of the adipose tissue using standard protocols.

Gene expression analysis by PCR

Total RNA was isolated from adipose tissue using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. RNA was transcribed into complementary DNA by reverse-transcription using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time PCR (qPCR) was performed using primers and power SYBR green master mix (Applied Biosystems, Foster City, CA) using the Stepone Real-Time PCR system (Applied Biosystems). Genes and primer sequences used in this study are listen in supplementary table 2. β-2-microglobulin was used as housekeeping gene for normalization procedures.

Gene expression analysis by microarray

RNA was isolated using TRizol reagent, and RNA quality was assessed using RNA 6000 nanochips on a Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Total RNA (100 ng), obtained from the CD14+ cells from five study participants was labeled with the Whole-Transcript Sense Target Assay (Affymetrix-ThermoFisher Scientific, Bleiswijk, the Netherlands; P/N 900652) and hybridized to whole-genome Affymetrix Human Gene 2.1 ST arrays. Sample labeling, hybridization to chips and image scanning was performed according manufacturer's instructions. Quality control and data analysis pipeline have been described in detail previously [26]. Briefly, normalized expression estimates of probe sets were computed by the robust multiarray analysis (RMA) algorithm [27] as implemented in the Bioconductor package *affyPLM*. Probe sets were redefined using current genome information according to Dai et al. [28] based on annotations provided by the Entrez Gene database, which resulted in the profiling of 29,526 unique genes (custom CDF v22). Differentially expressed probe sets (genes) were identified by using linear models (package *limma*) and an intensity-based moderated paired *t*-statistic [29, 30]. Subject-specific effects on expression were corrected for by blocking on subject in the design matrix. P-values were corrected for multiple testing by a false discovery rate (FDR) method [31].

Western blot

Abundance levels of CHIT1 protein were analyzed by western blot analysis. For sample preparation, adipose tissue lysates were generated from human adipose tissue using the milliplex map lysis buffer (Milliplex MAP, Millipore Corp, Billerica, MA). The lysates were diluted with 5% Laemmli sample buffer (3:1 v/v) and heated at 95 °C for 5 min followed by centrifugation at $2900 \times g$ at 4 °C for 2 min. Samples were loaded on a 10% polyacrylamide SDS-PAGE gel and electrophoresis was performed under reducing conditions. After SDS-PAGE, the proteins were transferred onto a PVDF membrane using 20% methanol in 25 mM Tris, 190 mM glycine as blotting buffer. The membrane was blocked for 1 h at room temperature with caseine blocking buffer (Sigma-Aldrich, Saint Louis, United States) diluted in PBS, according to manufacturer's guidelines, followed by overnight incubation at 4 °C with CHIT1 mouse monoclonal antibody (Santa Cruz biotechnology, Dallas, Texas, USA) diluted 1:1000 with 3% (w/v) BSA in TBS/Tween 20. After overnight incubation, the membrane was incubated with goat-anti mouse antibody (GAMPRO) (Dako, Glostrup, Denmark) diluted with 1:5000 5% (w/v) milk powder in TBS/Tween 20 for 2 h at room temperature. Finally, membrane was developed with enhanced chemiluminescence (ECL) (Thermo Scientific, Waltham, USA) according to manufacturer's guideline. Protein bands were visualized with Chemidoc XRS + (Biorad, Hercules, USA) and analyzed using Image Lab software (Biorad, Hercules, USA).

Plasma CHIT1 activity

The plasma enzyme activity of chitotriosidase was measured by a spectrofluorometric method as described previously [14]. In short, 5 µl of plasma was incubated with the substrate 4-methylumbelliferyl-β-D-N,N′,N″–triacetylchitotriose. The presence of chitotriosidase cleaves 4-methylumbelliferyl from the substrate and starts to fluoresce after adding the termination buffer (0.5 M glycine with 0.025% Trition X-100, pH 10.6). The activity of chitotriosidase was expressed as nanomoles of substrate hydrolyzed per milliliter per hour (nmol/ml/h).

Mutation analysis of *CHIT1* gene

Genomic DNA from adipose tissue was isolated from subjects across the three groups ($n = 95$), using the Qiagen

DNA extraction kit (Qiagen, Hilden, Germany). The 24 bp duplication site in exon 10 of the *CHIT1* gene was analyzed by specific primers ordered from Biolegio (Nijmegen, the Netherlands). DNA samples were mixed with a primer mix, forward and reversed primer prior to PCR. Subsequently, electrophoresis on a 4% agarose gel enabled detection of the frequent 24 bp duplication by discriminating a fragment of 75 and 99 bp from the normal and mutant *CHIT1* gene.

Statistical analysis

Data were reported as mean ± SD. Outliers were excluded by the descriptive analyses function using IBM SPSS statistics 22. Significant differences between groups were evaluated with ANOVA by GraphPad Prism 5 including a Bartlett's test for determining group variances. Relationships between variables were analyzed with Pearson correlation coefficients in MatLab (version 2014b, The MathWorks, USA). Pearson correlation coefficients were interpreted as: weak negative correlation ($-0.3 < R^2 < -0.3$), weak positive correlation ($0.3 < R^2 < 0.5$), moderate positive correlation ($0.5 < R^2 < 0.7$) or strong positive correlation ($R^2 > 0.7$) [32]. Differences after genotyping were analyzed with Mann–Whitney U tests in GraphPad Prism 5. A p-value lower or equal to 0.05 was considered significant for differences and correlations.

Multivariate analysis

Principal component analysis (PCA) was used as a screening method to identify patterns in the metadata. Partial least squares Discriminant Analysis (PLS-DA) was used to correlate the data to the class assignment of lean, overweight subjects and T2D patients. A repeated double cross validation procedure was performed to ensure a complete independent model, with a Leave-One-Out inner cross validation loop and a three-fold outer cross validation loop repeated 21 times [33]. The importance of CHIT1 mRNA levels, plasma CHIT1 activity and other metadata variables where determined using Variable Importance in Projection (VIP) method [34]. Auto scaling was performed for all multivariate models to avoid that variables with high values dominate the analyses. Model algorithms were performed in Matlab (version 2014b, The MathWorks, USA) with in-house written Matlab routines.

Results

Expression of CHIT1 in adipose tissue

First, the expression and localization of CHIT1 in human adipose tissue was evaluated. Mature adipocytes (MA) and the stromal vascular fraction (SVF), which contained the macrophages [35], were separated from paired human subcutaneous adipose tissue and visceral adipose tissue biopsies from seven subjects. As expected, CHIT1 mRNA levels were 65 times higher in the SVF compared to MA, correlating with the macrophage origin of CHIT1 (Fig. 1a). In the SVF, CHIT1 mRNA levels were 3.5 times lower in visceral adipose tissue compared to subcutaneous adipose tissue. Secondly, CHIT1 mRNA levels were also measured in isolated CD14+ cells from subcutaneous- and visceral adipose tissue and compared to expression in circulating CD14+ monocytes. CHIT1 mRNA levels were more than 20 times higher in CD14+ immune cells isolated from subcutaneous- or visceral adipose tissue (Fig. 1b). Thirdly, we determined abundance of CHIT1 protein in nine different adipose tissue lysates. In line with the data on gene expression level, western blot analysis revealed the presence of CHIT1 protein in all samples (Fig. 1c). Together, these data demonstrate expression of CHIT1 in adipose tissue-residing macrophages.

CHIT1 expression and plasma activity in overweight subjects and T2D patients

We then set out to evaluate whether CHIT1 abundance in adipose tissue correlates to parameters that reflect obesity, adipose tissue inflammation and T2D patients. To this aim, we used a cohort of 105 subjects, classified in three groups: lean, overweight and T2D. Subject characteristics of the study groups are shown in Table 1. As per definition, BMI, waist-to-hip-circumference (WHC), percentage of fat and fasting insulin levels were higher in overweight subjects compared to lean subjects whereas fasting glucose levels were higher in T2D patients. HbA1c was only measured in T2D patients. In addition, the available metadata of the study groups consisted of 22 parameters for all lean and overweight subjects and 23 parameters for all T2D patients. We determined, unsupervised, whether the metadata of the subject groups were discriminative. The variance present in the data was described by principal component analysis (PCA), which visualized a clear separation between lean, overweight subjects and T2D patients (Fig. 2), demonstrating the suitability of the subject groups for our research question on validation of CHIT1.

CHIT1 mRNA levels were subsequently measured in adipose tissue biopsies of 33 patients with T2D, and compared to the expression of the (non-diabetic) lean and overweight subjects ($n = 35$ and $n = 37$, respectively). CHIT1 mRNA levels were higher in overweight subjects compared to lean, but even further increased in T2D patients (Fig. 3a). To translate CHIT1 analysis to a format suitable in

Fig. 1 a Relative CHIT1 mRNA levels in mature adipocytes (MA) and stromal vascular fraction (SVF) of subcutaneous and visceral human adipose tissue (SAT and VAT respectively; $n = 7$). **b** Relative CHIT1 mRNA levels in CD14+ circulating cells and adipose tissue CD14+ cells isolated from SAT and VAT ($n = 5$). **c** Detection of CHIT1 in the area of interest in adipose tissue lysates from T2D individuals. Data are shown as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$
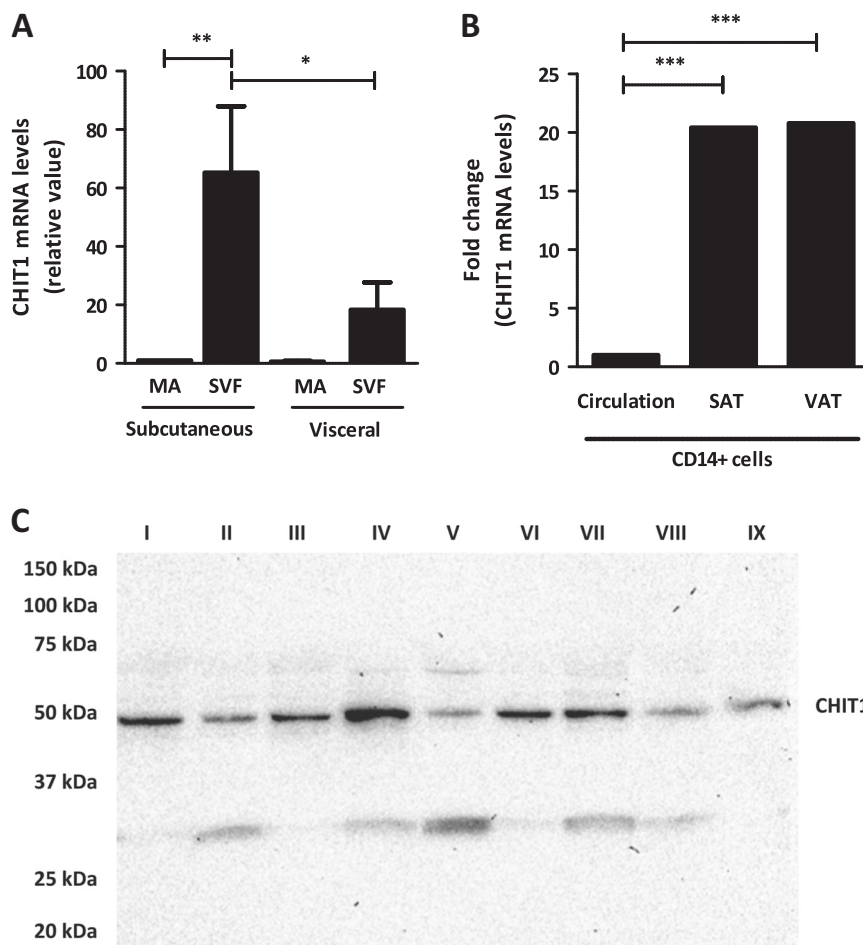

Table 1 Patient characteristics of lean, overweight and T2D subjects. Data are represented as mean ± SD, significance was defined as $p < 0.05$

	Lean controls ($n = 35$)	Overweight controls ($n = 37$)	Type 2 diabetes ($n = 33$)
Subject characteristics			
Gender (m/f)	17/18	17/20	19/14
Age (years)	54 ± 8	56 ± 9	61 ± 10
BMI (kg/m^2)	23.6 ± 1.8	31.4 ± 3.4[a]	29.3 ± 5.2
WHC	0.9 ± 0.1	1.0 ± 0.1[a]	1.0 ± 0.1
Fat %	16.6 ± 4.7	23.8 ± 6.3[a]	20.1 ± 6.9[b]
Fasting glucose (mmol/l)	5.1 ± 0.6	5.2 ± 0.6	11.3 ± 2.7[b]
Fasting insulin (μIU/ml)	10.6 ± 7.2	15.7 ± 9.7[a]	17.7 ± 6.1
HbA1c	—	—	8.6 ± 1.1

[a]Overweight subjects compared with lean subjects

[b]Type 2 diabetic subjects compared with overweight subjects

clinical practice, where options for adipose tissue sampling are limited, we evaluated a clinically usable CHIT1 assay measuring plasma CHIT1 enzymatic activity. Plasma CHIT1 activity levels are reported to be closely associated with plasma CHIT1 protein levels [36], allowing such translation. Plasma samples of all subjects of the three subject groups were tested for plasma CHIT1 activities. Figure 3b shows marked inter-individual variation in plasma CHIT1 activity, however, no marked differences between the three groups. In addition, no correlation was found between CHIT1 mRNA levels in the adipose tissue and plasma CHIT1 activity of all subjects (supplementary figure 1).
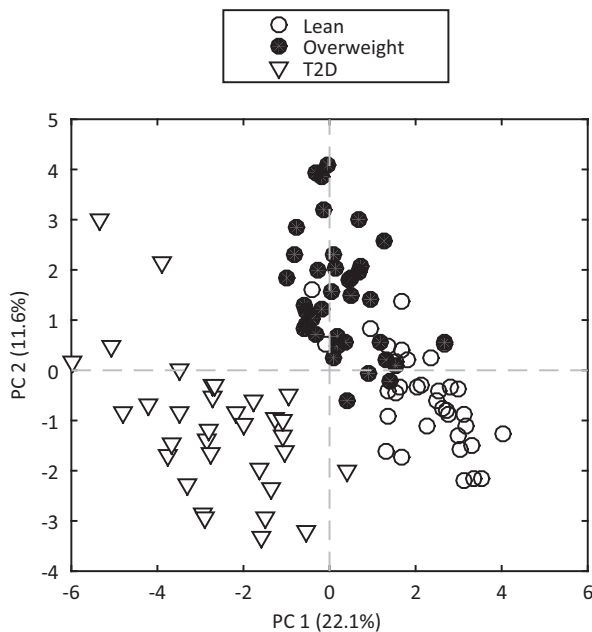
Fig. 2 Principal component analysis (PCA) describes the variance of the metadata and reveals a separation of lean, overweight subjects and type 2 diabetes patients

Genetic variation in CHIT1

A 24-bp duplication site in exon 10 in the *CHIT1* gene was reported to abolish plasma CHIT1 activity [37]. As this may affect our analysis, we assessed this genetic variation in CHIT1 in all subjects of who DNA was available, i.e. subjects with matched plasma- and DNA samples across the three groups ($n = 90$). Genotyping by PCR revealed that 62.1% of our subjects had no mutation, whereas 34.7% was heterozygous and 3.2% homozygous for the 24-bp duplication (see supplementary figure 2 for visualization of the 24-bp duplication analysis). These relative numbers correspond with earlier reports [39]. Plasma CHIT1 activity was reduced about half in heterozygous subjects as compared to wild-type subjects, whereas subjects homozygous for the 24-bp duplication did not show any plasma CHIT1 activity (Fig. 3c). Independent on the genetic variability, plasma CHIT1 activity was not significantly different between lean, overweight or T2D subjects that were separated based on their genotype (Fig. 3d).

Associations of CHIT1 with parameters of obesity and adipose tissue inflammation in lean and overweight subjects

Since CHIT1 mRNA levels in the adipose tissue were significantly higher in overweight subjects as compared to lean people (Fig. 3a), we evaluated how CHIT1 relates with obesity-related parameters and adipose tissue inflammation. CHIT1 mRNA levels were positively correlated to obesity-related parameters, viz. BMI, WHC and percentage of fat, and to adipose tissue inflammation parameters, viz. MCP-1 mRNA and protein levels (Fig. 4a). Plasma CHIT1 activity was positively correlated with IL-6 mRNA levels (Fig. 4b). In addition, 30.9% of the subjects had crown-like structures (CLS), macrophages that surround dead adipocytes, in their adipose tissue, which coincided with increased CHIT1 mRNA levels (Fig. 4c).

Associations of CHIT1 with parameters of T2D and adipose tissue inflammation in T2D patients

Next, we analyzed T2D patients and evaluated the relation between CHIT1 mRNA levels in adipose tissue and plasma CHIT1 activity with T2D-related parameters and markers of adipose tissue inflammation. CHIT1 mRNA levels were significantly correlated with fasting glucose levels and markers of adipose tissue inflammation, i.e. CD68 positive cells and MCP-1 mRNA (Fig. 5a) Plasma CHIT1 activity was correlated with MCP-1 mRNA levels (Fig. 5b). Surprisingly, those T2D patients that displayed CLS in their adipose tissue (36%) showed no increased CHIT1 mRNA expression in corresponding tissue samples (Fig. 5c). Addition of CHIT1 mRNA levels and plasma CHIT1 activities to the metadata variables did not contribute to the separation between the lean, overweight subjects and T2D patients in unsupervised PCA analysis (supplementary figure 3). Additionally, a supervised analysis using PLS-DA showed no contribution of CHIT1 mRNA levels and plasma CHIT1 activity to the discrimination between the lean, overweight subjects and T2D patients (data not shown).

Discussion

In this study, we evaluated whether CHIT1 levels, analyzed by both gene and protein expression in adipose tissue and by plasma activity levels, relate to adipose tissue inflammation and thereby could be of clinical value to discriminate between overweight individuals and T2D patients. We found higher expression in overweight subjects and T2D patients, but these differences did not translate to different plasma CHIT1 activity levels.

To our knowledge, we are the first to demonstrate the associations of CHIT1 with parameters of adipose tissue inflammation in lean, overweight, and T2D subjects. The accessibility of matched plasma and adipose tissue patient samples for this study enabled us to perform functional assays to evaluate the properties of CHIT1 on the level of adipose tissue and subsequently translate these findings to CHIT1 enzymatic activity in plasma. CHIT1 is expressed in monocytes and increased when these cells differentiate into macrophages [38–40] and is also secreted by macrophages
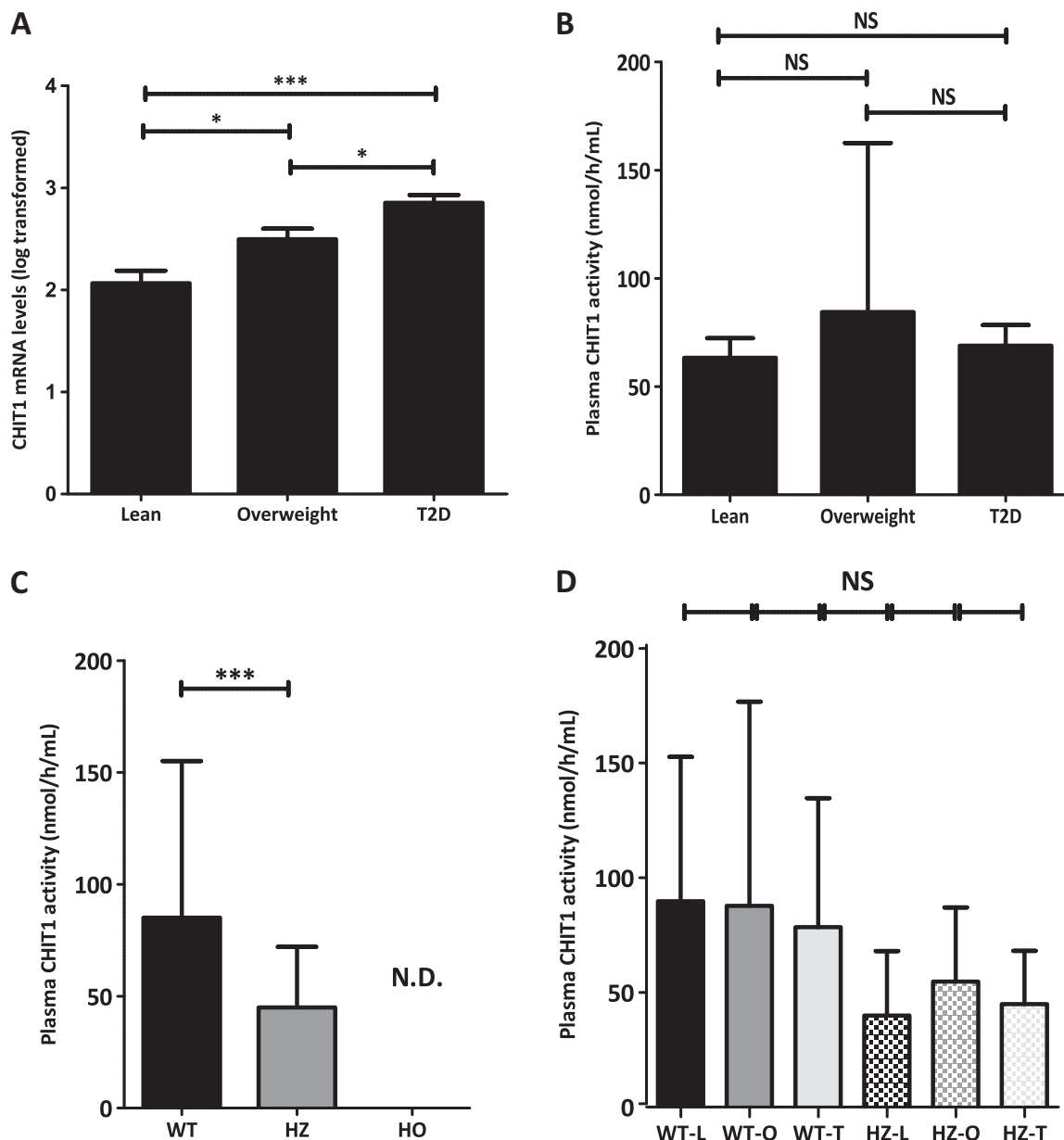
Fig. 3 a Analysis of CHIT1 mRNA levels in obtained subcutaneous adipose tissue of lean, overweight subjects and T2D patients ($n = 35$, 37, and 33, respectively). **b** Plasma CHIT1 activity measured in lean, overweight subjects and T2D patients ($n = 35$, 37, and 33 respectively). **c** Plasma CHIT1 activity for wild type (WT), $n = 55$, subject having no 24-bp duplication and thus no CHIT1 enzyme deficiency vs. subject that are heterozygous (HZ), $n = 32$, for the 24-bp duplication.

Subjects that are homozygous for the 24-bp duplication are not shown since they do not have CHIT1 enzyme activity. **d** Plasma CHIT1 activity for separated groups, *i.e.* WT lean (WT-L) $n = 17$, WT overweight (WT-O) $n = 19$, WT T2D (WT-T) $n = 19$, HZ lean (HZ-L) $n = 13$, HZ overweight (HZ-O) $n = 7$ and HZ T2D(HZ-T) $n = 12$. Data are shown as mean ± SD; p = not significant (NS) or *$p < 0.05$ or ***$p < 0.001$

[41], related to CHIT1's implication in inflammatory responses. Our results show that also in adipose tissue CHIT1 is mainly expressed in adipose tissue-residing monocytes and macrophages and increases from lean to overweight without T2D to (overweight) T2D. This is in line with previous findings in other tissues such as the liver, were CHIT1 was only expressed by liver-macrophages in patients with liver disease [42].

While obesity is associated with adipose tissue inflammation and insulin resistance, it has been suggested that a subset of obese people are relatively insulin sensitive and do not show signs of adipose tissue inflammation, sometimes referred to as "metabolically healthy obese" [43]. We find more pronounced adipose tissue inflammation and CHIT1-expression in patients with T2D compared to non-diabetic subjects with a similar level of obesity. This observation
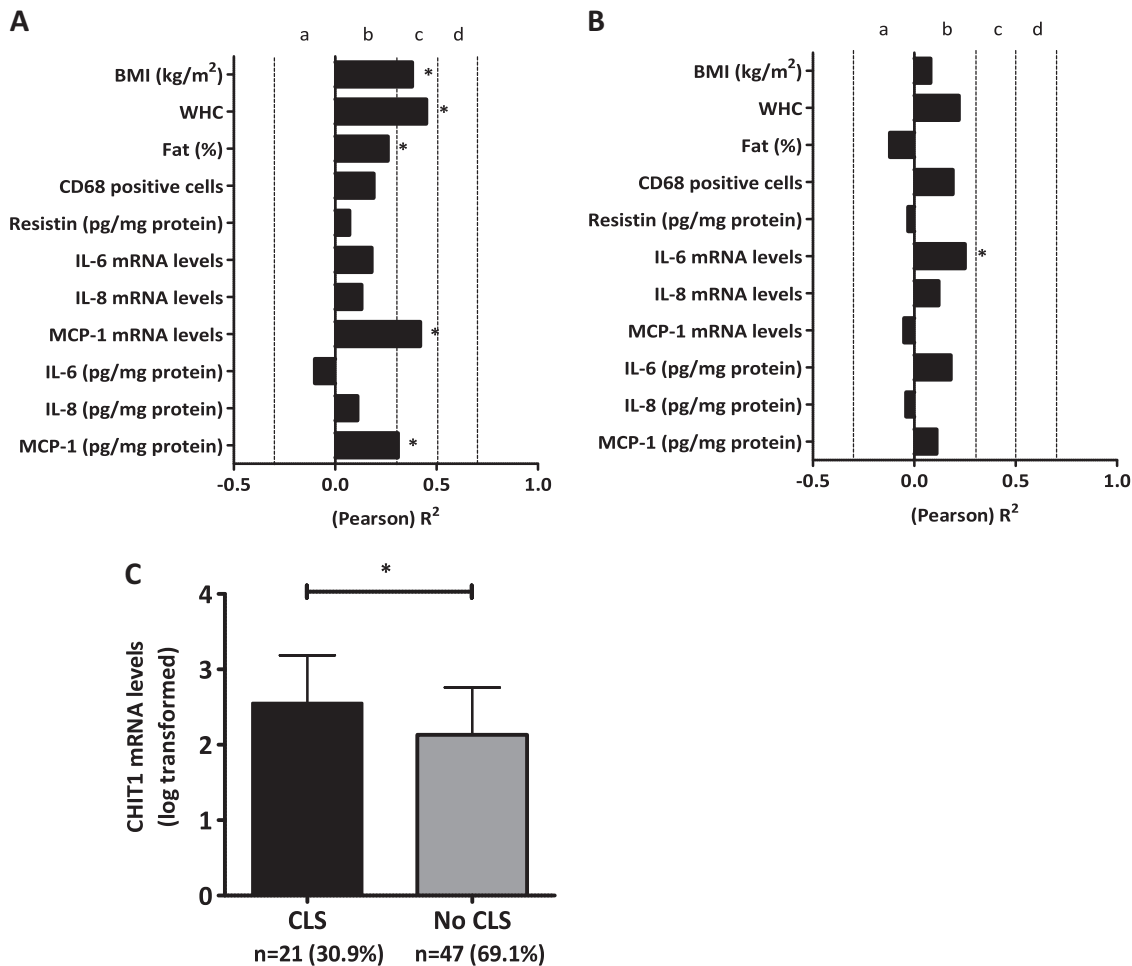
Fig. 4 Pearson correlation coefficient (R^2) of all parameters reflecting obesity and adipose tissue inflammation with CHIT1 mRNA levels (log-transformed) (**a**) and plasma CHIT1 activity (**b**) for all lean and overweight subjects ($n = 72$). A significant correlation was defined as $*p < 0.05$. **c** CHIT1 mRNA levels in subjects with and without CLS (crown-like structure). Data are expressed as mean ± SD; $*p < 0.05$. [a]Weak negative correlation. [b]Weak positive correlation. [c]Moderate positive correlation. [d]Strong positive correlation

may support this concept. However, the concept of metabolically healthy obese has also been criticized, as this "healthy" group still has an elevated cardiovascular risk [44] and over time most healthy become unhealthy obese [45]. The increased expression of CHIT1 in patients with T2D may also be related to the diabetic state as such or related to other factors.

To test our hypothesis that CHIT1 is a marker for adipose tissue inflammation, we examined CHIT1 mRNA levels and related them to parameters of adipose tissue macrophage numbers and activity. Obese adipose tissue inflammation is characterized by pro-inflammatory processes such as secretion of chemoattractants (e.g., MCP-1), infiltration of pro-inflammatory macrophages and the presence of CLS, which are clusters of macrophages that surround dead adipocytes [10, 46–49]. Within the adipose tissue, our results show that CHIT1 mRNA levels relate with MCP-1 mRNA and protein levels, but not with the

number of macrophages in lean and overweight subjects. This suggests that CHIT1 mRNA levels in adipose tissue are not a marker for (resident) macrophage numbers, but rather an indicator for migration of active macrophages into adipose tissue driven by MCP-1. Interestingly, preclinical studies show that the development of adipose tissue inflammation is first mediated by local proliferation of macrophages, while MCP-1-induced migration of macrophages only occurs when obesity proceeds and adipose tissue inflammation deteriorates [47, 49–52]. It is shown that MCP-1 is secreted into the extracellular space of adipocytes and cannot be detected in the circulation [49], which suggests that MCP-1's functions are limited to the adipose tissue [50]. Hence, the observed association with CHIT1 mRNA levels and MCP-1 mRNA levels in adipose tissue supports CHIT1 being a marker for aggravated adipose tissue inflammation. This is supported by the increased CHIT1 mRNA levels in overweight subjects with observed
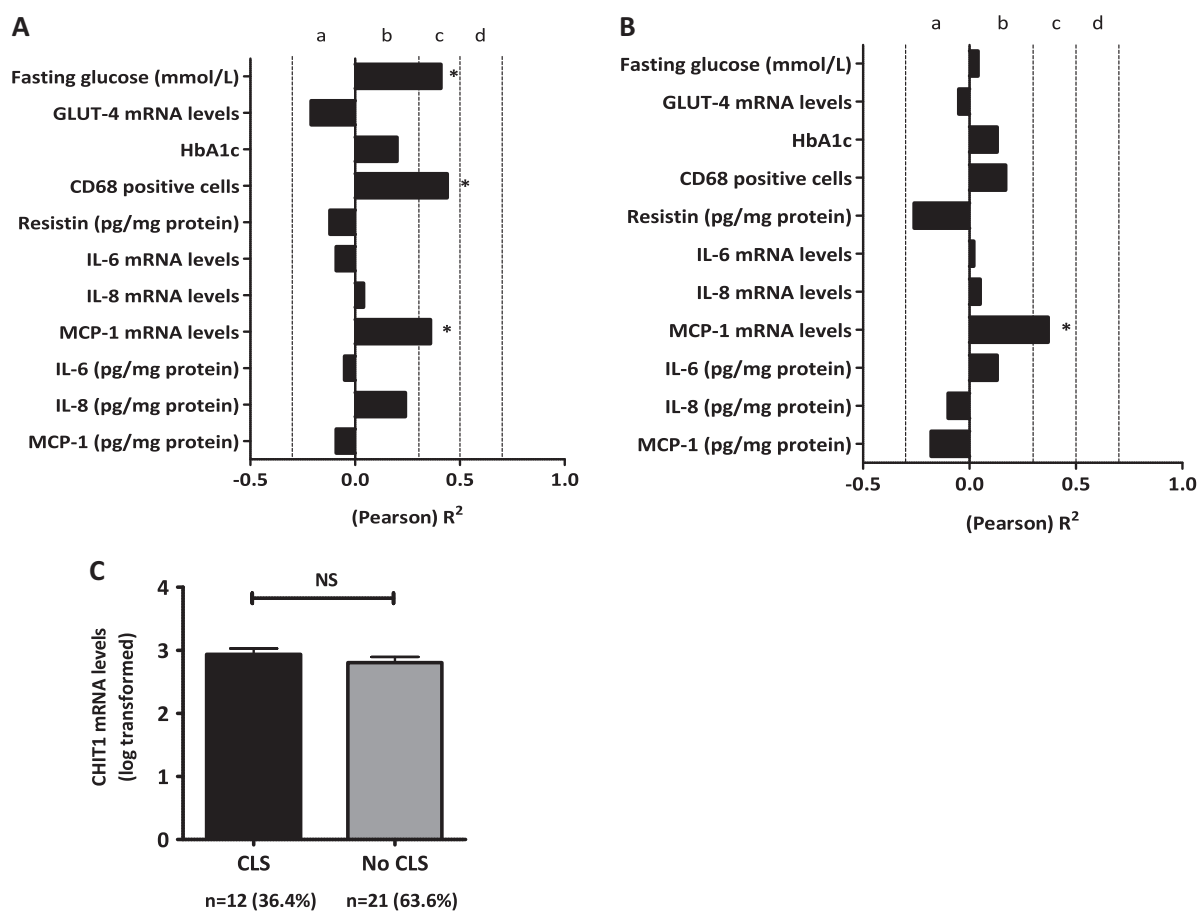
A

B

C

Fig. 5 Pearson correlation coefficients (R^2) of all parameters reflecting T2D and adipose tissue inflammation with CHIT1 mRNA levels (log transformed) (**a**) and plasma CHIT1 activity (**b**) for all T2D subjects ($n = 33$). A significant correlation was defined as $*p < 0.05$. **c** CHIT1 mRNA levels in subjects with and without CLS (crown-like structure). Data are expressed as mean $\pm$ SD; $p =$ not significant (NS). [a]Weak negative correlation. [b]Weak positive correlation. [c]Moderate positive correlation. [d]Strong positive correlation

crown-like structures, another reflection of aggravated adipose tissue inflammation. Interestingly, our data show the highest CHIT1 mRNA levels in adipose tissue of T2D patients and an association of CHIT1 mRNA levels with MCP-1 mRNA levels and the number CD68 positive cells. In contrast to lean and overweight subjects, it is plausible to assume that CHIT1 mRNA levels are a marker for both resident macrophage numbers and migration of macrophages into the adipose tissue. Increased MCP-1 mRNA levels promote infiltration of macrophages in the adipose tissue and subsequently induce insulin resistance [53], which on the long-term may result in T2D [54].

We did not observe any correlation between CHIT1 mRNA levels in adipose tissue and circulating plasma CHIT1 activity levels nor different plasma CHIT1 activity levels between lean, overweight subjects and T2D patients. There may be various reasons for this: (i) secretion of CHIT1 protein levels by the adipose tissue to the circulation is not substantial enough to observe consistent marked differences. (ii) Circulating CHIT1 protein activity

originates from more sources than adipose tissue only. (iii) CHIT1's biosynthesis and turnover in the adipose tissue was not investigated but may, if CHIT1's metabolism is high, result in lower secretion to the circulation.

Plasma CHIT1 activity levels in our study were not increased in T2D patients compared to lean and overweight individuals, while other studies show increased plasma CHIT1 activity in T2D subjects compared to controls [13, 17, 18, 55]. Plasma CHIT1 activity levels were observed to be higher in obese children compared to lean children but without further associations between CHIT1 and markers of obesity (e.g., insulin resistance) [56]. Similar to our findings, Turan et al. [57] observed no plasma CHIT1 activity differences between T2D subjects and controls. However, when subdividing T2D patients based on their complications (i.e., retinopathy, nephropathy, and heart failure), an increased plasma CHIT1 activity was found in T2D patients with complications as compared to patients without complications, and compared to controls [57]. Importantly, the T2D subjects from our study have no

such diagnosed complications nor were treated with insulin therapy. Thus, plasma CHIT1 activity may be a marker of complications of T2D rather than the presence of T2D itself. In line with this, Elmonem et al. [55] revealed that plasma CHIT1 activity in T2D patients was only increased in patients with micro- or macroalbuminuria, but not in T2D patients with normoalbuminuria.

A common mutation of the *CHIT1* gene is known to reduce plasma CHIT1 activity, which we confirmed in our analysis. However, studies reported in the literature do not always correct for the individual genotypes, which may thus interfere with average plasma CHIT1 activity levels that have been reported. The *CHIT1* gene consists of 12 exons, resides in chromosome 1q31-q32 and spans around 20 kilobases. A frequently occurring genetic deficiency results in a 24-bp duplication site in exon 10, yielding a CHIT1 protein lacking 29 amino acids at the C-terminal end of the protein, which mediates binding to chitin enabling CHIT1 to exerts its chitinolytic properties [12, 15, 58]. Six percent of the population is homozygous for this mutation while 35% carries one allele with the mutation. The mutation severely affects the CHIT1 enzyme activity [37, 58–60]. Elmonen et al. [55] reported a higher 24-bp duplication frequency in T2D patients with higher renal damage whereas Di Rosa et al. [61] found the lowest 24-bp duplication frequency in NASH patients compared to controls. We set out to determine how the CHIT1 genotype corresponds to plasma CHIT1 activity in the different groups and how this mutation affects the associations between parameters of obesity, adipose tissue inflammation and T2D. However, when separating groups based on the 24 bp duplication genotype, plasma CHIT1 activity was still not different between lean, overweight and T2D subjects. Despite this, the frequency of the *CHIT1* gene mutation should raise awareness once plasma CHIT1 activities are evaluated as a biomarker for any pathological condition. For future perspectives, it should be pointed out that plasma CHIT1 levels may be masked by this mutation and should therefore be taken into account.

We conclude that CHIT1 mRNA levels relate with adipose tissue inflammation as it is mainly expressed by innate immune cells resident in adipose tissue and positively associates with MCP-1 levels in overweight and T2D subjects. However, plasma CHIT1 enzyme activity levels do not correlate with adipose tissue inflammation markers which renders CHIT1 not suitable as a plasma biomarker for adipose tissue inflammation.

Compliance with ethical standards

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