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ORIGINAL ARTICLE

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Inflammation in the subcutaneous adipose tissue does not attenuate endothelial function in subjects with diabetes mellitus and subjects with dyslipidaemia and hypertension: A cross-sectional study

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Summary

Background: Obesity is associated with low-grade inflammation that may be related to vascular disease. We hypothesized that inflammation in the subcutaneous adipose tissue is associated with impaired endothelium-dependent vasodilatation.

Methods: We assessed endothelial function by measuring forearm vascular response to acetylcholine and determined inflammation in subcutaneous fat biopsies in 2 groups of subjects; 15 patients with type 2 diabetes mellitus (T2DM) and 19 subjects with dyslipidaemia combined with hypertension (DcH). The adipose tissue inflammation score was based on adipocyte size, influx of macrophages and presence of crown-like structures. We compared the vascular response to acetylcholine between subjects with and without adipose tissue inflammation.

Results: Patients with diabetes had clearly decreased vasodilatation compared to patients with DcH. In total, 23 of the 34 fulfilled the criteria of subcutaneous adipose tissue inflammation. However, there was no difference in vascular response to acetylcholine between the group with and without inflammation (changes in FBF from baseline 3.9 ± 0.8, 7.8 ± 1.0 and 13.6 ± 1.0 mL/dL/min compared to 4.3 ± 1.0, 7.9 ± 2.1 and 12.2 ± 2.4 mL/dL/min in response to acetylcholine 0.5, 2.0 and 8.0 μg/dL/min), nor was there a relationship between systemic hs-CRP levels and endothelial function.

Conclusions: We confirm that subjects with T2DM have impaired endothelial function compared to age- and BMI-matched subjects with DcH. However, endothelial function did not differ between participants with or without inflammation in the subcutaneous adipose tissue. These results suggest that fat tissue inflammation, at least in the subcutaneous compartment, does not affect vascular function.

KEYWORDS
cardiovascular disease, endothelial function, inflammation, subcutaneous fat tissue, type 2 diabetes mellitus

1 | INTRODUCTION

Obesity is characterized by low-grade inflammation which is linked to type 2 diabetes and atherosclerosis.[1] The enhanced inflammatory state in obese individuals has been suggested to originate from the expanded adipose tissue mass.

Obesity-induced inflammation is accompanied by several morphological changes in the adipose tissue. For instance, hypertrophy of adipocytes causes the production of cytokines and chemokines by the adipocytes.[2] In response to the raised production of the cytokines and chemokines, endothelial cells increase the expression of adhesion molecules. The release of chemokines and the increased expression of adhesion molecules recruit immune cells, including macrophages, to the adipose tissue.[3] Indeed, obesity is associated with infiltration of adipose tissue with macrophages in mice and human subjects.[4,5] In addition, enlargement of adipocytes leads to local hypoxia, which may result in adipocyte death that subsequently attracts macrophages to the adipose tissue[6] that cluster in crown-like structures (CLS) surrounding adipocytes that are dying. Noticeably, macrophages, part of CLSs, are known to display a more inflammatory profile as compared to individually dispersed macrophages in adipose tissue.[7]

Endothelial dysfunction is considered an early marker of vascular complications.[8,9] Endothelial dysfunction consists of a number of functional alternations in the vascular endothelium, including impaired endothelium-dependent vasodilatation which can be assessed by measuring responses to endothelium-dependent vasodilators.[10,11]

We hypothesize that inflammation in the subcutaneous adipose tissue impairs endothelium-dependent vasodilatation. To test this hypothesis, we assessed associations between inflammation in human adipose fat tissue and endothelium-dependent vasodilatation.

2 | MATERIALS AND METHODS

2.1 | Study population

This study represents a pathophysiological study embedded in 2 clinical trials in which subcutaneous fat biopsies were taken and endothelial function was assessed at the same time. Both studies were conducted in accordance with the principles outlined in the Declaration of Helsinki. The local ethics committee (Radboud University Medical Center, Nijmegen, the Netherlands) approved each study and all subjects gave written informed consent before participation. The study population consisted of 36 subjects who participated in previous trials. One study was a active treatment controlled investigating the effect of DPP-4 inhibitor vildagliptin on endothelial function in patients with type 2 diabetes mellitus.[12] Included were 16 subjects with type 2 diabetes mellitus who were treated with metformin with or without sulphonylurea or thiazolidinediones, aged 35-75 years old and had an HbA1c <8.0%. In this study, we refer to these participants as type 2 diabetes mellitus (T2DM).

The other study was a study on the effect of danshen on cardiovascular risk factors and the study population consisted of 20 subjects aged 40-70 years with dyslipidaemia and hypertension.[13] Included were subjects with a fasting LDL cholesterol >3.5 mmol/L and/or triglycerides >1.7 mmol/L. Excluded were triglycerides >8 mmol/L and/or LDL cholesterol >5 mmol/L or systolic blood pressure >180 mm Hg and/or diastolic blood pressure >110 mm Hg, respectively. This group of participants is referred to as dyslipidaemia combined with hypertension (DcH) in this study.

2.2 | Protocol

Both trials were double-blind, randomized, controlled, crossover trials. None of the studies showed carry-over effects. In this study, we used the data of the control arm of these trials. At the end of each treatment period, endothelial function was measured by venous occlusion plethysmography, a subcutaneous fat biopsy was taken and blood was collected for biochemical analysis.

2.3 | Experimental procedures

2.3.1 | Demographic and clinical characteristics

Patient demographics and use of medication were recorded. Body weight, height and blood pressure were measured.

2.3.2 | Biochemical analysis

Fasting blood samples were drawn to determine blood glucose levels, HbA1c, lipids and high sensitive C-reactive protein (hs-CRP) levels.

2.3.3 | Plethysmography

The experiments started at 8.30 AM after an overnight fast in a quiet, temperature-controlled room (23-24°C). The subjects abstained from caffeine for 24 hours prior to the experiment. The brachial artery of the nondominant arm (experimental arm) was cannulated for infusion of vasodilators and collection of blood samples.

Forearm blood flow (FBF) was measured using mercury-in-silastic strain gauge, venous occlusion plethysmography as previously described.[14] Forearm volume was measured with the water displacement method and all drugs were dosed per 100 mL forearm tissue with an infusion rate of 100 mL/dL/min.

After complete instrumentation, a 30-minute equilibration period was included, after which baseline measurements were performed with infusion of saline. Subsequently, 3 increasing doses of acetylcholine (0.5, 2.0 and 8.0 μg/dL/min, 10 mg/mL dry powder, dissolved to its final concentration with saline, Novartis, Greece) were infused into the brachial artery. Acetylcholine (Ach) stimulates endothelial muscarinic receptors thereby activating nitric oxide synthase. This results in the endothelial release of nitric oxide (NO) causing vasodilatation.[15] Each dose was infused for

TABLE 1 Baseline characteristics (mean ± SD)

Characteristic	T2DM (n = 15)	DcH (n = 19)
Age (y)	59.8 ± 7.0	57.9 ± 7.9
Sex (male:female)	11:4	13:6
Weight (kg)	88.5 ± 15.5	84.5 ± 17.4
BMI (kg/m^2)	29.0 ± 5.2	28.3 ± 5.6
Blood pressure systolic (mm Hg)	141 ± 8	156 ± 9***
Blood pressure diastolic (mm Hg)	82 ± 7	94 ± 5***
HbA1c (%)	6.9 ± 0.6	5.7 ± 0.3***
Total cholesterol (mmol/L)	4.4 ± 1.0	5.6 ± 0.8***
Triglycerides (mmol/L)	1.6 ± 0.8	1.8 ± 1.1
HDL cholesterol (mmol/L)	1.0 ± 0.2	1.2 ± .3
LDL cholesterol (mmol/L)	2.4 ± 1.0	3.5 ± 0.7***
Use of antihypertensives	7 (46%)	10 (50%)
Use of statins	11 (73%)	0 (0%)

DcH, dyslipidaemia combined with hypertension; T2DM, type 2 diabetes mellitus.
***P < .001 compared to subjects with type 2 diabetes mellitus.

5 minutes, and FBF was measured during the last 3 minutes of the 5-minute period. After the infusion of all 3 doses of acetylcholine, a 30-minute equilibration period is followed. Subsequently, baseline measurements were performed with the infusion of glucose 5% solution. Subsequently, 3 increasing doses of sodium nitroprusside (0.06, 0.20 and 0.60 μg/dL/min, 25 mg/mL, dissolved to its final concentration with glucose 5% solution, Clinical Pharmacy, Radboud University Medical Centre), an endothelium-independent vasodilator,[16] were infused in the brachial artery. Again, each dose was infused for 5 minutes and FBF was measured during the last 3 minutes of the 5-minute period. FBF registrations of the last 2 minutes of each dosage of vasodilator were averaged to a single value for data analysis.

2.3.4 | Subcutaneous adipose tissue biopsy

A subcutaneous adipose tissue biopsy was taken from the abdominal region after completion of the plethysmography. Biopsies were obtained under local anaesthesia (2% lidocaine HCl), from an area about 10 cm lateral of the umbilicus using a Hepafix Luer lock syringe (Braun, Melsungen, Germany) and a 2.10 × 80 mm Braun medical Sterican needle (Braun). Adipose tissue was washed using a 0.9% normal saline solution. The adipose tissue was snap frozen and stored at −80°C until further analysis, or it was fixed in 4% paraformaldehyde and embedded in paraffin for morphometric analysis.

Morphometry of individual fat cells was assessed using digital image analyses as described previously.[17] For each subject, the adipocyte cell size of all fat cells in ten microscopic fields of view (magnification 10×) were counted and measured. Adipocyte size was expressed as area (μm^2), perimeter (μm), feretmin (μm) which is the smallest diameter and feretmax (μm) which is the largest diameter.

An antibody against CD68 (AbD Serotec, Kidlington, UK) was used to stain macrophages. Macrophage influx was quantified by counting the number of adipocytes and macrophages in ten representative microscopic fields of adipose tissue (magnification 40×).

Adipose tissue inflammation was based on a composite score consisting of fat cell size, number of CD68+ cells/adipocyte and the presence of crown-like structures (CLS). The score uses means as cut-off points within the population as a whole and of the respective subpopulations. For subjects with T2DM, the score consisted of feretmin >74 μm, CD68+ cells/adipocyte >0.12 and the presence of CLS. For subjects with DcH, the score consists of feretmin >67 μm, CD68+ cells/adipocyte >0.12 and the presence of CLS. The inflammation score was positive if a participant fulfilled at least 1 out of the 3 criteria of inflammation. We included a group of healthy controls to document increased inflammatory levels in adipose tissue in the presence of T2DM or DcH.

2.4 | Statistical analysis

All data are represented as mean ± SEM unless otherwise indicated. Differences in means of laboratory results were tested by Student's t test or Mann-Whitney test for non-normally distributed data. Correlations were calculated by Pearson correlation coefficient. Two-tailed P < .05 was considered significant. NS indicates nonsignificant.

FBF was expressed as absolute change in FBF (ΔFBF) above baseline. Analysis of variances (ANOVA) was used to assess differences in increments in FBF between groups (T2DM vs DcH, inflammation vs no inflammation). Statistical analyses were performed using GRAPHPAD 5.0.

3 | RESULTS

A total of 36 participants who completed both trials were included in the study, resulting in 34 paired subcutaneous fat biopsies and endothelial function measurements. One participant excluded from statistical analysis because of unreliable results of the plethysmography, and 1 participant did not provide informed consent to perform a subcutaneous fat biopsy. Baseline characteristics of the 34 participants included in the analyses are shown in Table 1.

3.1 | T2DM vs DcH

The 2 groups did not differ in age, BMI and gender. Due to different inclusion criteria, the subjects differed in HbA1c levels, blood pressure and lipids (Table 1).

In subjects with T2DM, endothelium-dependent vasodilatation was decreased compared to DcH. Changes in FBF from baseline in T2DM were 2.0 ± 0.7, 5.0 ± 1.2 and 11.7 ± 1.6 mL/dL/min in response to acetylcholine dosage 0.5, 2.0 and 8.0 μg/

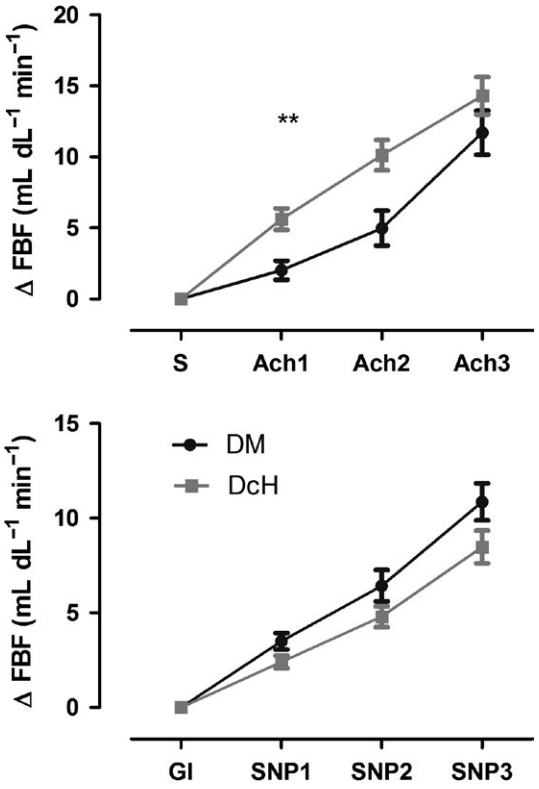

FIGURE 1 Vascular response to endothelium vasodilators (top) and endothelium-independent vasodilators (bottom) in subjects with type 2 diabetes mellitus (T2DM) compared to subjects with dyslipidaemia and hypertension (DcH). ACH, acetylcholine; SNP, sodium nitroprusside. ***P* = .009 by two-way ANOVA

dL/min, respectively, compared to 5.6 ± 0.8, 10.1 ± 1.1 and 14.3 ± 1.3 mL/dL/min in DcH (*P* = .009 by two-way ANOVA) (Figure 1—top).

There was no difference in response to sodium nitroprusside between subjects with T2DM and DcH. Changes in FBF from baseline in T2DM were 3.5 ± 0.4, 6.4 ± 0.8 and 10.9 ± 1.0 mL/dL/min in response to sodium nitroprusside dosage 0.06, 0.20 and 0.60 μg/dL/min, respectively, compared to 2.4 ± 0.3, 4.8 ± 0.5 and 8.5 ± 0.9 mL/dL/min in DcH (*P* = NS). (Figure 1—bottom).

3.2 | Subcutaneous adipose tissue inflammation

Compared to BMI-matched controls, the influx of macrophages in the subcutaneous adipose tissue, as measured by CD68+ cells/adipocyte, was clearly increased in both T2DM and DcH (0.13 in T2DM, 0.12 in DcH vs 0.03 in healthy controls).

Out of the 34 participants, 23 scored positive on the inflammation score. There was no difference in vascular response to acetylcholine between the group with and without inflammation. Changes in FBF from baseline in the inflammation group were 3.9 ± 0.8, 7.8 ± 1.0 and 13.6 ± 1.0 mL/dL/min in response to acetylcholine 0.5, 2.0 and 8.0 μg/dL/min, respectively, compared to 4.3 ± 1.0, 7.9 ± 2.1 and 12.2 ± 2.4 mL/dL/min without inflammation (*P* = NS) (Figure 2A).

3.2.1 | Subgroup analyses

Of the 15 subjects with T2DM 10 fulfilled the criteria for inflammation in the subcutaneous adipose tissue (8 fulfilled the criterium of feretmin >74 μm, 8 had CD68/adipocyte ratio >0.12 and 4 had CLS).

No difference in vascular response to acetylcholine between the group with and without inflammation was observed. Changes in FBF from baseline in the inflammation group were 2.5 ± 0.9, 6.3 ± 1.6 and 13.1 ± 1.9 mL/dL/min in response to acetylcholine 0.5, 2.0 and 8.0 μg/dL/min, respectively, compared to 1.1 ± 0.8, 2.3 ± 1.2 and 8.8 ± 2.3 mL/dL/min without inflammation (*P* = NS) (Figure 2B).

Likewise, inflammation score was positive in 13 out of the 16 subjects with DcH, again with no difference in endothelium-dependent vasodilatation between the group with and without inflammation (9 fulfilled the criterium of feretmin >67 μm, 6 had CD68/adipocyte ratio >0.12 and 3 had CLS).

Changes in FBF from baseline in the inflammation group were 5.6 ± 1.0, 10.0 ± 1.1 and 14.5 ± 1.2 mL/dL/min in response to acetylcholine 0.5, 2.0 and 8.0 μg/dL/min, respectively, compared to 5.6 ± 1.2, 10.3 ± 2.6 and 13.9 ± 3.5 mL/dL/min without inflammation (*P* = NS) (Figure 2C). Adipose tissue macrophages can cluster in crown-like structures (CLS) that possess a high inflammatory status. Out of the 34, 7 participants displayed CLS in the subcutaneous adipose tissue biopsy material. There was no difference in endothelium-dependent vasodilatation between the group with and without the presence of CLS. Changes in FBF from baseline were 4.1 ± 1.7, 7.5 ± 2.0 and 10.9 ± 2.2 mL/dL/min in response to acetylcholine 0.5, 2.0 and 8.0 μg/dL/min, respectively, in the group with CLS compared to 4.0 ± 0.6, 7.9 ± 1.0 and 13.7 ± 1.1 mL/dL/min in the group without CLS (*P* = NS) (Figure 2D).

Mean circulating hs-CRP level, a marker of systemic inflammation, was 1.98 ± 0.39 μg/mL (n = 34). Hs-CRP level did neither correlate with the influx of macrophages in the subcutaneous fat tissue (CD68 positive cells) nor with endothelial function and area (Figure 3). As expected, hs-CRP levels were significantly correlated with measures of obesity (BMI and the area of adipocytes) and with HOMA-IR.(data not shown).

4 | DISCUSSION

The present study confirms that subjects with type 2 diabetes mellitus have impaired endothelial function compared to age-, gender- and BMI-matched subjects with dyslipidaemia combined with hypertension. Both T2DM and DcH have increased inflammation in the subcutaneous adipose tissue compared to controls. However, there was no relationship between adipose tissue inflammation and endothelial (dys) function. This conclusion is based on the observation that endothelium-dependent vasodilatation did not differ between the subjects with inflammation and without inflammation in adipose tissue. Furthermore, there was no association between hs-CRP levels and vascular function.

Endothelial dysfunction is regarded as an important marker of vascular complications in type 2 diabetes mellitus.[8] Hyperglycaemia, hypercholesterolaemia, obesity and hypertension all negatively

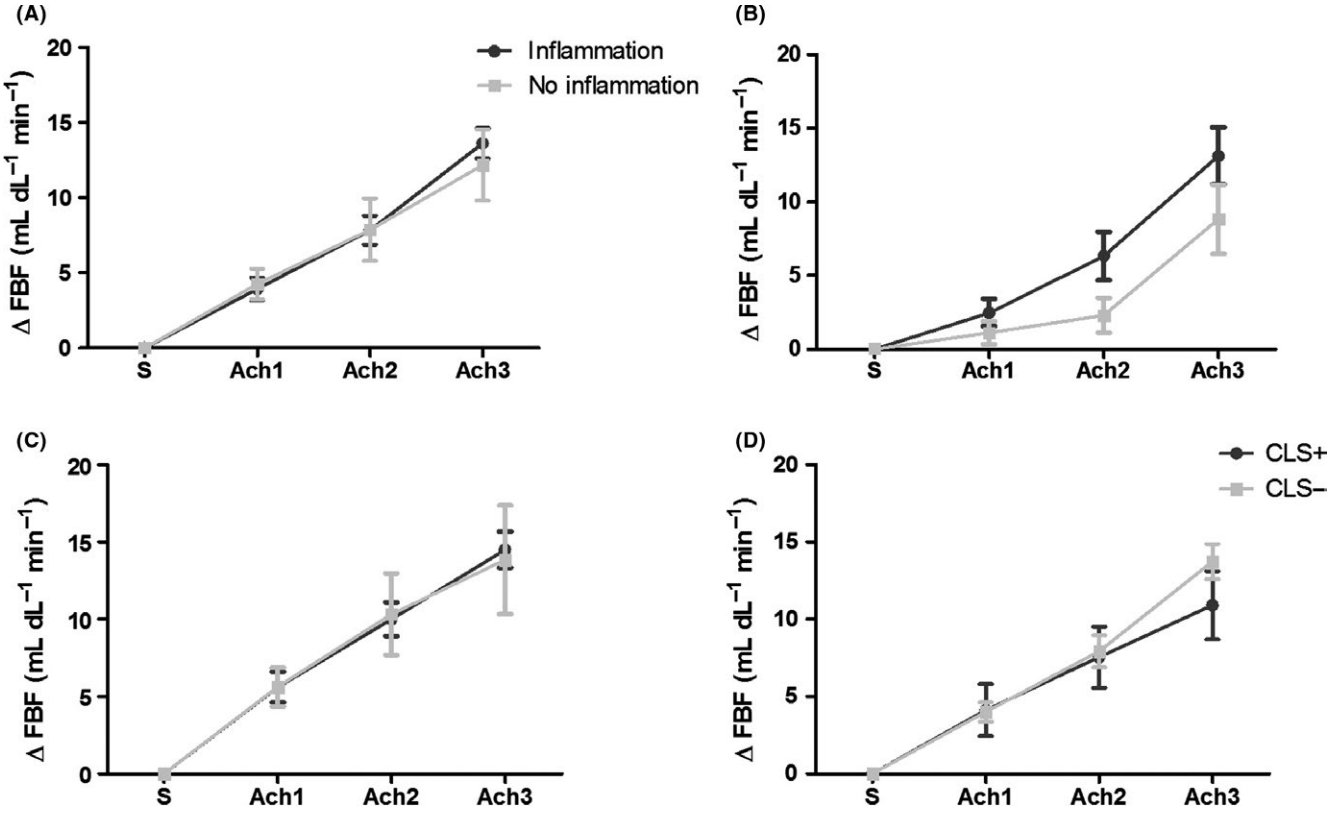

FIGURE 2 Relation between inflammation in subcutaneous adipose tissue and endothelium-dependent vasodilatation. Change in forearm blood flow in the experimental arm in response to acetylcholine (dosage 0.5, 2.0, 8.0 μg/dL/min) in (A) both trials in the inflammation group (n = 23, black) and the group without inflammation (n = 11, grey), (B) in subjects with type 2 diabetes mellitus with inflammation (n = 10, black) and without inflammation (n = 5, grey) and (C) in subjects with both dyslipidaemia and hypertension with inflammation (n = 13, black) and without inflammation (n = 6, grey). Finally, in graph D, the change in forearm blood flow in the experimental arm in response to acetylcholine is shown for subjects with crown-like structures (CLS) in adipose tissue biopsy (n = 7, black) and those without (n = 27, grey). ACH, acetylcholine; SNP, sodium nitroprusside

affect endothelial function.[18] In the present study, we confirm that subjects with type 2 diabetes mellitus have impaired endothelium-dependent vasodilatation compared to subjects with both dyslipidaemia and hypertension, 2 well-known risk factors for cardiovascular disease. This is in line with the well-known increased cardiovascular disease risk associated with type 2 diabetes and underlines that this increased risk is partly independent from other risk factors.[19,20]

In this study, we were able to associate detailed vascular responses with adipose tissue inflammation in a fairly large group of subjects with diabetes and subjects with cardiometabolic risk factors. The study did not reveal an impaired endothelial-dependent vasodilatation in subjects with inflammation in subcutaneous adipose tissue as compared to those without inflammation whatsoever. These results seem to be in contrast with 2 previous studies showing that the presence of CLS in adipose tissue of obese subjects is associated with endothelial dysfunction.[21,22] There are important differences in population and methodology that may explain these diverging findings. One study in obese subjects shows that the presence of CLS in the subcutaneous fat compartment is associated with impaired endothelial function, as measured by flow-mediated vasodilatation (FMD)[21] but the population in this study consisted mainly of morbidly obese individuals. Farb et al[22] demonstrate that obese subjects with inflamed fat, defined as the presence of CLS,

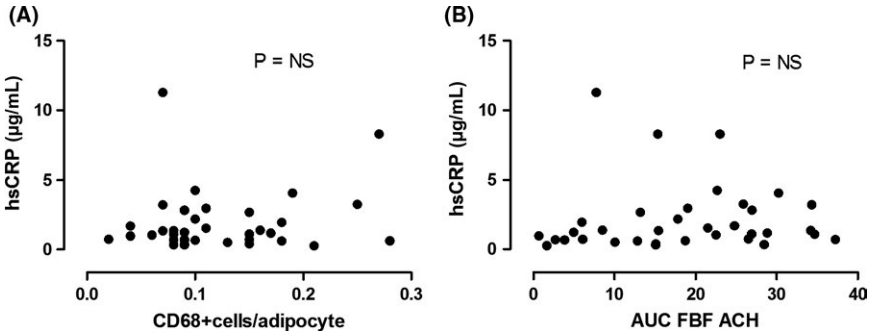

FIGURE 3 Relation between hs-CRP levels and inflammation of subcutaneous adipose tissue (A) and endothelium-dependent vasodilatation (B). ACH, acetylcholine; AUC, area under the curve; FBF, forearm blood flow

showed lower FMD compared to lean subjects and obese subjects without inflamed fat. Again, obese subjects in this study consisted of morbidly obese subjects many of whom subsequently underwent bariatric surgery. The lean control group was also younger. Interestingly, the percentage of patients with diabetes did not differ between the obese subjects with or without adipose tissue inflammation in both studies.

In our study, the population investigated was moderately overweight/obese, with a mean BMI of approximately 29 kg/m^2. A second relevant difference with the earlier studies is that we used vascular responses to acetylcholine to assess endothelial function, while in the other studies, FMD was used. These different measures might have led to different results. Zeiher et al[23] demonstrated that 3 methods of measuring endothelial function in the coronary system, that is, FDD, cold pressor test and infusion of acetylcholine, produced different results in patients with different stages of atherosclerosis. This implies that the degree of atherosclerosis influences the results of testing endothelial function. Intra-arterial acetylcholine infusion measures vascular responses in the forearm resistance vessels, while FMD measures the response of conduit artery (a. brachialis). Although it is tempting to conclude that intra-arterial infusion of endothelium-dependent vasodilators represents a more direct and reproducible parameter of endothelial function, ultimately, both response to acetylcholine and FMD are negatively associated with cardiovascular outcomes.[10]

Finally, in the present study, we used an overall inflammation score, while the other studies only used the presence of CLS. There is no clear definition of adipose tissue inflammation. We used a scoring system including adipocyte size, macrophages influx and presence of CLS to categorize inflammation. However, if we analysed subjects with and without CLS, there still was no difference in endothelium-dependent vasodilatation. Percentage-wise, the number of subjects with CLS was clearly lower than those in the earlier studies, perhaps related to the less extreme level of obesity. Taken together, adipose tissue inflammation may be relevant for vascular dysfunction in the extreme end of obesity, but of relatively minor importance in the intermediate obesity range and in the context of other cardiometabolic risk factors such as diabetes, dyslipidaemia and hypertension.

As fat tissue inflammation is more pronounced in the visceral fat compartment,[24] our findings do not exclude a potential relationship between visceral fat inflammation and endothelial function. So far, no studies have reported on the relationship between visceral fat inflammation and functional vascular measurements.

C-reactive protein is a marker of low-grade inflammation and a risk marker for cardiovascular disease as it has been shown to predict cardiovascular events.[25,26] In vitro studies have shown that CRP decreases NO bioactivity in endothelial cells by downregulation of endothelial nitric oxide synthase (eNOS).[27,28] Given the effect that CRP has on NO biology one would expect that high CRP levels are associated with impaired endothelial function. Our study shows no relation between CRP levels and vascular response to acetylcholine. Studies in healthy subjects have shown conflicting results with regard to the association between CRP levels and impaired endothelial.[29,30] In subjects with coronary artery disease, Fichtlscherer et al[31] showed that elevated CRP levels were associated with endothelial dysfunction.

It is important to underline that our study also has limitations. First, the cross-sectional design provides only of associations between inflammation and endothelial function, but provides no information on the sequence of events; therefore, causality cannot be assessed. Second, we used data from the control arm of 2 different studies, which enabled us to compare endothelial function between T2DM and DcH. The 2 groups were comparable with regards to age, BMI and gender, but differed in lipid levels, blood pressure and use of medication (T2DM 44% used antihypertensives and 68% used statins, DcH 47% used antihypertensives and no statins were used). Despite lower blood pressure and lower lipid levels, T2DM still had impaired endothelial function.

In summary, we confirm that subjects with T2DM have impaired endothelial function compared to age- and BMI-matched subjects with DcH, despite lower lipid and blood pressure levels in the diabetes group. This finding emphasizes the increased risk of cardiovascular complications inherent to type 2 diabetes mellitus. The present study did not find a difference in endothelial function between the groups of participants with and without inflammation in the subcutaneous adipose tissue. These results suggest that fat tissue inflammation in patients with moderate overweight/obesity, at least in the subcutaneous compartment, does not affect vascular function.

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CONFLICT OF INTEREST

Nothing to declare.

AUTHORS' CONTRIBUTIONS

PCMP contributed to study design, collected the data, analysed the data and wrote the manuscript. EJA and RS contributed to study protocol and manuscript. MN contributed to the manuscript. CT contributed to study design, analysed the data and contributed to the manuscript.

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