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# Acute exercise increases plasma levels of muscle-derived microvesicles carrying fatty acid transport proteins.

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Abbreviations:

EV; extracellular vesicle

CD36, cluster of differentiation 36

FATP4; fatty-acid transport protein 4

LCFA; long-chain fatty acid

MV; microvesicle

SkMV; skeletal muscle-derived microvesicle

SR-B2, scavenger receptor B2

Context: Microvesicles (MVs) are a class of membrane particles shed by any cell in the body in physiological and pathological conditions. They are considered to be key players in intercellular communication, and with a molecular content reflecting the composition of the cell of origin, they have recently emerged as a promising source of biomarkers in a number of diseases.

Objective: The effects of acute exercise on the plasma concentration of skeletal muscle-derived microvesicles (SkMVs) carrying metabolically important membrane proteins were examined.

Methods and Results: Thirteen obese male patients with type 2 diabetes mellitus (T2DM) and 14 obese healthy male controls exercised on a cycle ergometer for 60 min. Muscle biopsies and blood samples obtained before exercise, immediately after exercise, and 3-h into recovery were collected for the analysis of long-chain fatty acid (LCFA) transport proteins CD36 (SR-B2) and FATP4 mRNA content in muscle, and for flow cytometric studies on circulating SkMVs carrying either LCFA transport protein. Besides establishing a novel flow cytometric approach for the detection of circulating SkMVs and subpopulations carrying either CD36 or FATP4, and thereby adding proof to their existence, we demonstrated for the first time an overall exercise-induced change of SkMVs carrying these LCFA transport proteins. A positive correlation between exercise-induced changes in skeletal muscle CD36 mRNA expression and concentrations of SkMVs carrying CD36 was found in T2DM only. Conclusions: This approach could add important real-time information about the abundance of LCFA transport proteins present on activated muscle cells in subjects with impaired glucose metabolism.

Effect of acute exercise on levels of muscle-derived microvesicles carrying long-chain fatty acid transport proteins was investigated. Increased levels were observed in T2DM and obese individuals.

## Introduction

Carbohydrate and fat are dominant substrates for skeletal muscle metabolism and during exercise there is a complex interaction between skeletal muscle fat and carbohydrate metabolism (1,2). A major source of fat is long-chain fatty acids (LCFAs) from adipose tissue (3). Cellular LCFA uptake most likely takes place either by passive diffusion through the lipid bilayer, or is facilitated by membrane-associated proteins, or by a combination of both. In muscle, transport of LCFAs is mediated by several transport proteins, including the widely expressed transmembrane glycoprotein, cluster of differentiation 36 (CD36), a scavenger receptor class B protein (SR-B2), plasma membrane-associated fatty-acid binding protein (FABPpm), and fatty-acid transport proteins 1 and 4 (FATP1 and FATP4) (4-7). Transport protein-mediated LCFA uptake is a key step in cellular fatty acid utilization, and impaired regulation of this process may lead to intracellular triacylglycerol accumulation and cellular insulin resistance (IR) (8). In obesity, accumulation of intra-myocellular triglyceride (IMTG) is positively associated with IR. However, it seems unlikely that IMTG cause IR directly, but rather protects cells from IR by preventing the accumulation of lipotoxic intermediates such as diacylglycerol (DAG) and ceramide (9,10), each of which thought to engage serine kinases that disrupt the insulin signaling cascade, thereby causing IR (11).

In rat skeletal muscle, CD36 and FATP4 are the most effective LCFA transport proteins *in vivo* (12). The acute effects of a single bout of moderate exercise on skeletal muscle *CD36* and *FATP4* mRNA levels were previously described in rodents (13,14) and humans (5). However, studies of LCFA transport protein content and function have been hampered by the limited amount of skeletal muscle tissue obtained by percutaneous muscle biopsies in humans. Thus, most LCFA transport protein studies during acute exercise on humans are on either giant vesicles or crude membrane extracts of total homogenates (15,16).

It is well known that skeletal muscle produces myokines during physical activity (17,18) and that these signaling molecules cover a whole range of auto-, para- and endocrine effects (19,20), suggesting a molecular link between muscle function and whole body physiology. Moreover, the finding that microRNAs (miRNAs) are secreted in a similar manner into the bloodstream during muscle-contraction (21), suggests an extensive cross-talk between muscle and other tissues. In the past decade, extracellular vesicles (EVs) have been recognized as potent vehicles of intercellular communication due to their capacity to transfer proteins, lipids and nucleic acids, as reviewed in (22). EVs are released from the surface of various cell types and based on their biogenesis or release pathways, they are often divided into exosomes (40

to 120 nm), microvesicles (MVs) (100 to 1000 nm) and apoptotic bodies (50 nm to 2 μm) (23,24). The recent findings that exosomes carrying miRNAs are shed from muscle tissue after a bout of acute exercise (25,26) underlines the extraordinary role of skeletal muscle as a secretory organ important for intercellular metabolic communication. To what extent MVs are released from skeletal muscle in the resting state, and whether the release of MVs are augmented in response to acute exercise remains to be established.

Inspired by the growing body of data implicating an exercise-induced release of vesicles we hypothesize that skeletal muscle-derived MVs (SkMVs) carrying important molecular information are released during exercise and affected by mRNA transcription levels and by the presence of T2DM. Thus, more specific our aims were to establish the presence of circulating SkMVs, and to investigate the release of SkMVs during a single bout of exercise. MVs are surrounded by plasma membrane from their parental cell, and thus LCFA transport protein content in SkMVs during acute exercise may reflect adaptations *in vivo*. On this background, we aimed to determine concentrations of SkMVs carrying CD36 and FATP4. Skeletal muscle IR in type 2 diabetes mellitus (T2DM) is characterized by impaired insulin signaling, increased intramyocellular fat content and mitochondrial defects, as reviewed in (27). Both LCFA uptake and plasma membrane LCFA transport protein content have been shown to be higher in skeletal muscle with IR (28) and adaptable to exercise (29,30). Thus, we additionally sought to investigate whether T2DM affected the release of SkMVs expressing LCFA transport proteins in the resting state or in response to acute exercise. Our third aim was to study the potential of SkMVs as a surrogate to tissue biopsy-based biomarkers by investigating whether exercise-induced changes in muscle transcription levels of *CD36* and *FATP4* mRNA are reflected in SkMVs carrying these transport proteins.

## Materials and Methods

### Study subjects

In the present study, we investigated skeletal muscle biopsies and blood samples obtained before, immediately after, and 3 h after an acute bout of endurance exercise. The study population, including medication details, eligibility criteria, clinical and metabolic characteristics have been described previously by Pedersen et al. (31,32). In brief, thirteen obese male patients with T2DM and 14 obese healthy male control individuals, matched for age, BMI and physical activity levels, participated in the study (Table 1). Informed consent was obtained from all participants before participation. The study was approved by The Regional Scientific Ethical Committees for Southern Denmark and was performed in accordance with the Helsinki Declaration.

### Study design

One week before exercise day, all participants underwent exercise tests to determine maximal aerobic capacity ($VO_{2max}$) as previously reported (31-33). On the exercise day, participants were required to exercise on an ergometer (Monoark Ergomedic 839 E, Vansbro, Sweden) for 60 min at power outputs that corresponded to approximately 70% $VO_{2max}$, which is considered to be a moderate to high intensity exercise training. Muscle biopsies and fasting venous blood samples were obtained 20 min before exercise (pre-exercise), immediately after 60 min of exercise (post-exercise) and after a post-exercise recovery period of 180 min (recovery) (Fig. 1). All medications were withdrawn one week prior to the study day, and participants were instructed to abstain from exercise 48 h before the exercise test.

### MV analysis

Blood samples were collected at pre-exercise, post-exercise, and recovery and platelet-poor plasma (PPP) was prepared by centrifugation (2100 x g, 10 min., 8°C), immediately frozen, and stored at – 80° C until analysis. For each analysis, 50 $\mu$L of freshly thawed PPP was

transferred to a TruCount™ tube (BD Biosciences, New Jersey, USA) containing a known number of fluorescent beads used for calculating MV concentrations. Subsequently, SkMVs were labeled by adding 5 $\mu$L fluorescein isothiocyanate- (FITC-) conjugated Lactadherin (83 $\mu$g mL−1, Haematologic Technologies Inc., Vermont, USA) characterized by a phosphatidylserine- (PS-) bonding motif, followed by 5 $\mu$L Phycoerythrin- (PE-) conjugated anti-human muscle-specific sarcolemmal beta-Sarcoglycan (480.0 $\mu$g mL$^{-1}$ $IgG_{2A}$, $\kappa$ (dilution: 1:5; Abcam, Cambridge, UK), and either 8 $\mu$L Allophycocyanin- (APC-) conjugated anti-human FATP4 (10.0 $\mu$g mL$^{-1}$ $IgG_{2B}$ (clone #342142, R&D Systems Europe Ltd., Abington, UK)) or 15 $\mu$L APC-conjugated anti-human CD36 (6.25 $\mu$g mL$^{-1}$ IgM, $\kappa$ (clone CB38, BD Pharmingen, New Jersey, USA)). After 30 min. of incubation (4°C, in the dark), 250 $\mu$L 0.22-$\mu$m filtered PBS was added to each labeled sample. MVs were analyzed by flow cytometry using a BD FACSAria™ III High Speed Cell Sorter equipped with BD FACSDiva™ software (v. 6.1.3) and three air-cooled lasers (488 nm, 633 nm and 407 nm). Using fluorescence threshold triggering (in the blue part of spectrum) to discriminate fluorescently labeled vesicles from non-fluorescent noise, as described in recent studies (34,35), we were able to detect fluorescent 100-nm silica beads on the basis of both fluorescence and scatter properties (FSC-H/SSC-H). A size-defined MV region (100-1000 nm) was established in a FSC-H/SSC-H setting (log scale) using a blend of fluorescent 100-nm and 1000-nm silica beads (Kisker Biotech GmbH & Co. KG, Germany). Logarithmic amplification was used for all channels and isotype ab controls added to Lactadherin-FITC-stained plasma samples were used as negative controls. Results were analyzed using FlowJo™ (v. 10, Tree Star, Inc., Oregon, USA) software.

**RNA isolation and cDNA synthesis**

Skeletal muscle biopsies were obtained from the vastus lateralis muscle at pre-exercise, post-exercise and recovery as described in (32). Total RNA was extracted from skeletal muscle biopsy using the TRIzol protocol (Applied Biosystems/Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions and as described previously (36). Total RNA was treated with DNaseI (Amplification Grade, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems/Life Technologies, Foster City, CA, USA).

**Quantitative real time PCR**

Quantitative real-time PCR (qRT-PCR) was performed on a Mx3005P® QPCR System instrument (Stratagene/Agilent, CA, USA) using the following pre-designed TaqMan® Gene Expression Assays (Applied Biosystems/Life Technologies, Foster City, CA, USA): CD36 (Hs00169627_m1), FATP4 (SLC27A4) (Hs00192700_m1), PPIA (Hs04194521_s1), and B2M (Hs00984230_m1). All samples were run in triplicates. The mRNA levels of *CD36* and *FATP4* were normalized to the geometric mean of *PPIA* and *B2M*. Data were analyzed using qBase+ software (Biogazelle, Zwijnaarde, Belgium) (37,38).

**Statistics**

Statistical evaluation was performed using STATA 11.2 (StataCorp LP, Texas, USA). All data were tested for normality. Normally distributed data were described using mean and standard deviation (SD), and non-parametric data were described using median and interquartile range (IQR). Parametric data were compared using a Student's t-test (unpaired, 2 tails) test and non-parametric data using a Mann–Whitney U test or Wilcoxon test as appropriate. The linear dependence between two groups of data was assessed by Spearman´s rank correlation coefficient. $P \leq 0.05$ was considered statistically significant.

## Results

**Identification of SkMVs and SkMVs expressing LCFA transport proteins by flow cytometry.** Circulating SkMVs were identified as lactadherin binding phosphatidylserine-positive particles expressing muscle-specific beta-Sarcoglycan, a 43 kDa dystrophin-associated glycoprotein and integral component of the dystrophin-glycoprotein complex. The contour plot in Fig. 2a shows the presence of PS+ SkMVs < 1 µm in diameter. The contour plot in Fig. 2b represents the negative control (plasma vesicles labelled with Lactadherin-FITC and a PE-conjugated beta-Sarcoglycan-matched isotype control).

By the initial finding of circulating SkMVs, we aimed to identify SkMVs carrying CD36 and FATP4. The contour plots in Fig. 3 (a-d) show the presence of (a) CD36+ and (c) FATP4+ SkMVs when compared to the respective matched isotype controls shown in (b) and (d).

**Acute exercise increases the release of SkMVs expressing LCFA transport proteins.** To investigate the influence of a single bout of exercise on circulating SkMVs expressing LCFA transport proteins, we compared their concentrations in plasma collected during exercise and recovery. Pre- and post-exercise levels of total SkMVs (median-values) were 13% (p=0.065) and 27% (p=0.047) higher, respectively, in T2DM patients, compared to obese controls, but comparable at recovery. Levels of SkMVs carrying any of the two LCFA transport proteins were comparable at all three time points, when comparing T2DM patients with obese controls (Table 2).

Total SkMVs were unaffected by exercise in both study groups except at recovery, where SkMV levels were slightly reduced in patients with T2DM (p=0.033), when compared to post-exercise levels (Table 2). Interestingly, post-exercise levels of CD36+ SkMVs increased by 52% (p=0.019) in T2DM patients, and by 55% (p=0.016) in obese controls (Table 2 and Fig. 4a), and FATP4+ SkMVs increased by 53% (p=0.007) in T2DM patients, but were unchanged in obese controls (Table 2 and Fig. 4b). The recovery period had no effect on SkMVs carrying any of the two LCFA transport proteins, when compared to post- or pre-exercise levels.

**Muscle mRNA transcripts correlate with changes in levels of CD36+, but not FATP4+ SkMVs.** Skeletal muscle *CD36* and *FATP4* mRNA expression levels were comparable at all three time points, when comparing the two study groups. At recovery, *CD36* mRNA expression was reduced by 25% in T2DM patients (p=0.047), when compared to the post-exercise state, otherwise post-exercise and recovery had no effect on LCFA gene expression (Fig. 5). Our next aim was to examine to what extent the variation of the number of circulating SkMVs carrying LCFA transport proteins is determined by variation of the corresponding mRNA in muscle. By comparing changes in gene expression and SkMVs carrying LCFA transport proteins concentrations, we found correlation between exercise-induced changes of *CD36* expression and concentrations of SkMVs carrying CD36 (Rho=0.65, p=0.032). This correlation was only found post-exercise and only in T2DM patients. In contrast, changes in *FATP4* mRNA expression and concentrations of SkMVs carrying FATP4 were unrelated in both study groups following exercise and recovery.

## Discussion

To the best of our knowledge, this is the first study to directly measure circulating SkMVs and subpopulations of SkMVs carrying metabolically important membrane proteins released into the circulation during exercise. Previous studies have demonstrated the presence of small skeletal muscle-derived EVs or exosomes released *in vitro* from immortalized murine C2C12 myoblast lines (39,40) and *in vivo* into the human bloodstream (25,26,41). In the present study we established a flow cytometric method for measuring SkMVs and the subpopulation of SkMVs expressing LCFA transport proteins.

The validity and reproducibility of the current used methodology has previously been described (42). Because of their small size, EVs are below the detection range of most conventional flow cytometers (43). However, by using fluorescence threshold triggering instead of scatter on a dedicated high-sensitivity flow cytometer enabled us to increase the detection sensitivity. Moreover, by labeling cellular vesicles directly in plasma we demonstrate a simple, sensitive and low-cost method to directly measure and phenotyping plasma vesicles with no additional isolation steps prior to vesicle quantification. There were several novel observations: (a) post-exercise levels of CD36+ SkMVs were increased in both study groups, whereas only patients with T2DM showed increased post-exercise levels of FATP4+ SkMVs, and (b) a positive correlation between exercise-induced changes in skeletal muscle *CD36* mRNA expression and concentrations of CD36+ SkMVs was observed in T2DM patients, but not in obese controls. Contraction of skeletal muscle fibers increases the levels of circulating exosome/nano-sized vesicles (25,26,39), and a very recent study by Whitham and coworkers demonstrated an exercise-induced increase in several classes of proteins associated with small vesicles and exosomes using nano-ultra-high-performance liquid chromatography (UHPLC) tandem mass spectrometry (41). It was thus somewhat unexpected that we did not find elevated post-exercise levels of SkMVs. However, MVs and exosomes are generated and released by different mechanisms and stimuli, and thus cannot readily be compared. Furthermore, studies of exosomes like (25,26,41) have several drawbacks. One is the purification methods, which introduce loss of material as well as co-precipitation of other non-exosome contaminants (44,45). Another is that studies are performed on the bulk of exosomes and thus increased exosomes cannot directly be ascribed to release by skeletal muscle. The method introduced in the present paper allow quantification of MV directly in plasma without prior purification steps, and further, by measuring MVs derived from skeletal muscle, we obtain results which can be directly related to exercise effect on skeletal muscle fibers. As another novel finding, we demonstrate for the first time an overall exercise-induced increase of SkMVs expressing important LCFA transport proteins. Increased LCFA oxidation during exercise is facilitated by a rapid and sustained upregulation of LCFA uptake by predominantly CD36 and FATP4 (7,14,46), and even short-term exercise increases LCFA uptake in both isolated muscle preparations and exercising study subjects (47). Thus, since total SkMV levels were unchanged, the increased levels of SkMVs expressing LCFA transport proteins indicate that these vesicles could mirror real-time expression levels on parental skeletal muscle cells, increased translocation from intracellular pools to the sarcolemma, or both.

Both insulin stimulation and exercise increase LCFA uptake in muscles via translocation of LCFA transport proteins to the plasma membrane (13,48-51), and the finding that especially CD36 migrates to the membrane without an increase in total protein content of CD36 in obese rats (52) emphasizes the importance of measuring LCFA transport proteins directly in the muscle plasma membrane and not simply in whole muscle homogenate. Thus, herein, we introduce a methodological approach based on highly sensitive flow cytometry which enable us to detect and quantitate LCFA transport protein-positive SkMVs shed from activated skeletal muscle cells. Providing important real-time information about the abundance of LCFA transport proteins present on activated muscle cells could potentially add new insight into skeletal muscle physiology.

We measured levels of CD36+ and FATP4+ SkMVs at three selected time points and found no difference among our study groups, thus supporting earlier studies showing increased but equal plasmalemmal CD36 content in muscle from T2DM and obese individuals (53,54). Although similar studies on plasmalemmal FATP4 are missing in the literature, we expected comparable levels in obesity and T2DM, as reported for similar LCFA

transport proteins, including FABPpm and FATP1 (55). However, further investigations should address this issue.

Although, we did not directly measure sarcolemmal abundance of LCFA transport proteins, our observation of increased post-exercise levels of SkMVs carrying CD36 and FATP4 suggests an increased sarcolemmal abundance of the two transport proteins. This is in agreement with previous reports on increased CD36 and FATP4 translocation to the sarcolemma in response to muscle contraction (56,57). However, the finding of increased post-exercise levels of FATP4+ SkMVs in T2DM patients, but not obese controls, needs further investigation. Potentially, an impaired glucose metabolism combined with lower muscle glycogen content in T2DM (58) could drive the demand towards using LCFAs as fuel. A higher plasma membrane content of LCFA transport proteins may therefore provide a cellular mechanism through which rates of LCFA uptake are increased in skeletal muscle with IR (53). Although the biological function of the results presented herein remains unclear, and further investigation is needed in this regard, we suggest a link between increased concentrations of SkMVs carrying LCFA transport proteins and the higher need for LCFAs to support the training-induced increase in skeletal muscle FA oxidation.

Expression levels of *CD36* and *FATP4* mRNAs were comparable in our study groups at all time points, thus in line with a previous study investigating skeletal muscle mRNA levels of LCFA transport proteins in obesity and T2DM (55). A single bout of exercise had no significant effect on LCFA gene expression levels, as previously reported by others (59). Transcription and translation can be differently regulated and others have observed that the mRNA abundance of LCFA transport proteins in adipocytes of obese rodents shows poor correlation with LCFA transport protein expression and LCFA transport (52,60-62). Moreover, studies on skeletal muscles report that an increase in sarcolemmal CD36 (51,53) is accompanied by a decrease in intracellular CD36 and not by an increase in total *CD36* expression (54). Herein we report a positive correlation between changes in skeletal muscle *CD36* mRNA expression and concentrations of CD36+ SkMVs, but only in patients with T2DM. We can only speculate about the lack of correlation between *FATP4* mRNA levels and protein levels on circulating SkMVs, and why the correlation of CD36 was restricted to T2DM, though the dissimilarity could be caused by mechanisms related to muscle IR. CD36 and FATP4 protein content in skeletal muscle are stimulated by insulin and contraction (7,13,63,64), however, CD36 seems to be stimulated in an additive manner, suggesting separate insulin- and contraction-sensitive intracellular depots for CD36, but not for FATP4 (56,60). Thus, although both transcription and translation may be coordinately regulated, we cannot rule out the influence of possible intracellular trafficking pathways and/or additional protein regulation at the level of protein degradation. Taken together, results must be interpreted with care and more studies are still needed to fully understand these findings.

The current study design is particularly valuable because it permits a sensitive detection and quantification of skeletal muscle-derived vesicles carrying LCFA transport proteins. Moreover, comparing skeletal muscle mRNA expression with protein abundance on SkMVs has not previously been done – especially not in human muscle with IR. Although a lean control group could have added additional important information to the study, our primary goal was to investigate whether T2DM, but not obesity, affected levels and composition of SkMVs. As for weaknesses of our study, a larger number of participants, as well as inclusion of women to rule out gender-effects, are needed to draw any conclusions about relationships. Moreover, we did not take into account other demographic factors, such as smoking and excessive alcohol consumption.

As another possible weakness, we used venous blood instead of blood from a femoral artery, which is in closer proximity to the vesicle releasing tissue. Thus, we cannot rule out that released vesicles are taken up by resident cells or in the liver, as recently demonstrated

by Whitham el at. (41). Although a direct comparison of protein abundance in muscle membrane and SkMVs would have been beneficial, our method is a step towards a simpler approach to investigating membrane protein abundance *in vivo* and could be complementary to other methodologies as well. Finally, FATP4 is expressed predominantly in skeletal muscle tissue (4,5,63,65), whereas beta-Sarcoglycan and CD36 is expressed in skeletal, cardiac, and smooth muscle (66-69). Thus, there is a possibility of contributions from other muscle tissue besides skeletal muscle.

Whether or not the release of vesicles is part of tissue crosstalk and/or simple an evolutionary conserved process in which tissues can share resources during the high energy demands of physical exertion, as suggested by Whitman et al.,(41) needs to be addressed in future studies. However, previous studies show that skeletal myocyte cultures release and take up EVs (70,71). Both uptake and release of EVs are energy-dependent processes. Thus, it seems very unlikely that horizontal transfer of vesicle cargo is a random process. Although protein transfer may not be as critical as transfer of regulatory RNAs it may still be beneficial for recipient cells to receive fully functional proteins. When it comes to quantitating protein abundance on skeletal muscle membranes we do not believe that the method presented herein is a better or more precise measure, compared to subcellular fractionation or other plasma membrane isolations methods. However, we demonstrate a simple, effective and time-saving method that hold potential to noninvasively give information on protein abundance on specific tissue.

In summary, the novel data presented herein not only demonstrate the presence of circulating SkMVs, but also add proof to the existence of SkMVs carrying transport proteins important in LCFA uptake, of which we observed an increase during exercise.

Furthermore, this study is the first to report on *CD36* and *FATP4* expression levels in skeletal muscle with IR during acute exercise and recovery, and to suggest a possible link between skeletal muscle mRNA levels and protein content on circulating SkMVs.

The results presented herein provide no physiologic or mechanistic insight to exercise-induced secretion of vesicles nor the impact of healthy versus insulin resistant state. Thus, more clarifying studies are still needed. Nevertheless, the ability to gain important real-time information about the abundance of LCFA transport proteins present on activated muscle cells by studies of circulating SkMVs could potentially add new insight into skeletal muscle physiology.

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Data availability

Data are available on request from the authors.

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Contribution statement

MHN, KH and AH were responsible for the conception and design of the study. MHN performed cytometry analysis in collaboration with FACS Core Facility, The Faculty of Health, Aarhus University, Denmark, and wrote the first version of the manuscript.

RS performed qRT-PCR analysis. AJTP and KH recruited the study participants. MHN contributed to analysis of data. MHN and AH contributed to the interpretation of the results.

All authors revised the manuscript and approved the final version. MHN and AH are responsible for the integrity of the work as a whole.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Disclosure Summary:

The authors have nothing to declare.

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Fig. 1. (legend). Schematic representation of the study protocol. Skeletal muscle biopsies and fasting venous blood samples were obtained 20 min before exercise, immediately after 60 min of cycle ergometry at ~70% $VO_{2max}$, and after a recovery period of 180 min.

Fig. 2. (legend). Contour plot of circulating SkMVs. (a) SkMVs detected using fluorescence threshold triggering were labelled with FITC-conjugated Lactadherin and PE-conjugated anti-human muscle-specific beta-Sarcoglycan. (b) As a negative control, plasma samples were stained with FITC-conjugated Lactadherin and a PE-conjugated beta-Sarcoglycan-matched isotype control.

Fig. 3. (legend). Contour plots of SkMVs expressing LCFA transport proteins. (a) SkMVs expressing CD36 were labelled with an APC-conjugated anti-human CD36 antibody and compared with (b) a matched isotype control. Similarly, (c) SkMVs expressing FATP4 were labelled with an APC-conjugated anti-human FATP4 antibody and compared with (d) a matched isotype control.

Fig. 4. (legend). Impact of a single bout of exercise on circulating SkMV levels in plasma. Results are presented in scatter dot plots (logarithmic scale, median with interquartile range). Plasma levels of (a) CD36+ SkMVs, and (b) FATP4+ SkMVs, during exercise and recovery in T2DM patients and obese controls. *p<0.05.

Fig. 5. (legend). Expression of CD36 and FATP4 mRNAs in skeletal muscle biopsies obtained at pre-exercise, post-exercise and recovery: (a) total skeletal muscle CD36 mRNA, (b) total skeletal muscle FATP4 mRNA. mRNA levels were measured by RT-qPCR and values presented as bar charts with individual data points (means ± SEM). a.u., arbitrary units. *p<0.05.

Table 1. Characteristics of participants.

| Characteristics | Controls | T2DM |
|---|---|---|
| n | 14 | 13 |
| Age (years) | 55±2 | 55±2 |
| BMI ($kg/m^2$) | 29.0 ± 0.9 | 29.7±1.0 |
| Fasting plasma glucose (mmol/l) | 5.6±0.1 | 10.0±0.7*** |
| $HbA_{1c}$ (mmol/mol) | 37±4.0 | 53 ± 7.9*** |
| $HbA_{1c}$ (%) | 5.5±0.1 | 7.0±0.2*** |
| Plasma cholesterol (mmol/l) | 5.7±0.3 | 4.4 ± 0.4* |
| Plasma LDL-cholesterol (mmol/l) | 3.8±0.3 | 2.0±0.2** |
| Plasma HDL- cholesterol (mmol/l) | 1.3±0.1 | 1.2 ± 0.1 |
| Plasma triacylglycerol (mmol/l) | 1.5±0.2 | 3.2 ± 1.5 |
| $VO_{2max}$ (l/min) | 3.50 ± 0.17 | 3.22±0.23 |
| $W_{max}$ (W) | 236±12 | 196±20 |
| Diabetes duration (years) | - | 3.5 ± 1.2 |

Data are means ± SEM
*p<0.05, **p<0.001 and ***p<0.0001 (Controls vs. T2DM)
$W_{max}$, maximal workload capacity

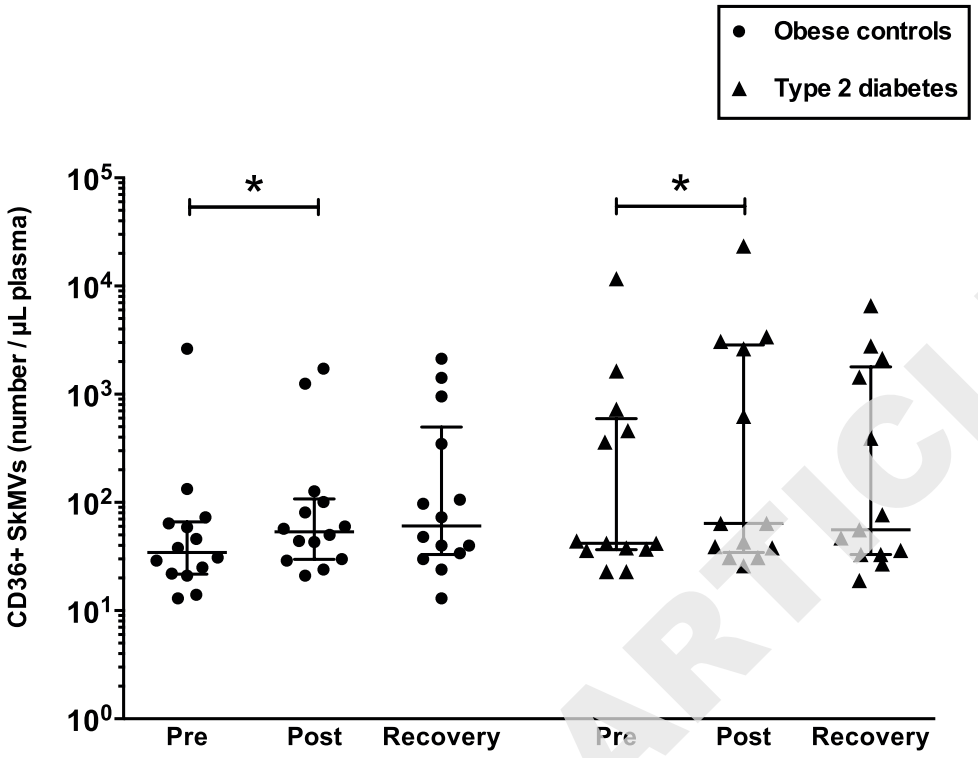
Table 2. Circulating SkMVs at baseline, after an exercise bout and during recovery.

| | Total SkMVs | CD36+ SkMVs | FATP4+ SkMVs |
|---|---|---|---|
| Controls (n=14) | | | |
| Pre-exercise | 1156 (773-1207) | 34.5 (22-64) | 103.5 (20-530) |
| Post-exercise | 994 (737-1551) | 53.5 (30-101)* | 63 (18-351) |

| | | | |
|---|---|---|---|
| Recovery | 1058.5 (846-2153) | 60.5 (34-347) | 108 (33-1242) |
| T2DM (n=13) | | | |
| Pre-exercise | 1309 (1011-2548) | 42 (37-462) | 49 (15-788) |
| Post-exercise | 1259 (1013-8180)$^{‡}$ | 64 (38-2631)* | 75 (22-1654)* |
| Recovery | 1184 (1032-6691)$^{\#}$ | 56 (33-1442) | 51 (35-1015) |

Concentration of SkMVs, SkMVs expressing CD36 and SkMVs expressing FATP4 were measured in diabetic patients and obese controls. Values (SkMVs $\mu L^{-1}$ plasma) are shown as the median (interquartile range). SkMVs, skeletal muscle-derived microvesicles; CD36, fatty acid translocase/scavenger receptor CD36; FATP4, Fatty acid transport protein 4. *p<0.05, compared to pre-exercise; $^{\#}$p<0.05, compared to post-exercise, $^{‡}$p<0.05, T2DM compared to obese controls.

a




b