1 **Title:** The effects of aerobic exercise training at two different intensities in obesity and type 2

2 diabetes: Implications for oxidative stress, low grade inflammation and nitric oxide

3 production

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20 (nitric oxide metabolites); T2DM (type 2 diabetes mellitus); CRP (C-reactive protein).

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27   **ABSTRACT**

28   **Aims:** To investigate the effect of 16 weeks of aerobic training performed at two different
29   intensities on nitric oxide (tNOx) availability and iNOS/nNOS expression, oxidative stress
30   (OS) and inflammation in obese humans with or without Type 2 Diabetes Mellitus (T2DM).
31   **Methods:** Twenty-five sedentary, obese (BMI >30kg/m^2) males (52.8±7.2 yrs); 12 controls
32   vs. 13 T2DM were randomly allocated to four groups that exercised for 30 minutes, three
33   times per week either at low (Fat-Max; 30-40% VO_{2max}) or moderate (Tvent; 55-65%
34   VO_{2max}) intensity. Before and after training, blood and muscle samples (v. lateralis) were
35   collected. **Results:** Baseline erythrocyte glutathione was lower (21.8±2.8 vs. 32.7±4.4
36   nmol/mL) and plasma protein oxidative damage and IL-6 were higher in T2DM (141.7±52.1
37   vs. 75.5±41.6 nmol/mL). Plasma catalase increased in T2DM after Tvent training (from
38   0.98±0.22 to 1.96±0.3 nmol/min/ml). T2DM groups demonstrated evidence of oxidative
39   damage in response to training (elevated protein-carbonyls). Baseline serum tNOx were
40   higher in controls than T2DM (18.68±2.78 vs. 12.34±3.56 μmol/L). Training at Tvent
41   increased muscle nNOS and tNOx in the control group only. Pre-training muscle nNOS was
42   higher in controls than in T2DMs, while the opposite was found for iNOS. No differences
43   were found after training for plasma inflammatory markers. **Conclusion:** Exercise training
44   did not change body composition or aerobic fitness, but improved OS markers, especially
45   when performed at Tvent. Non-diabetics responded to Tvent training by increasing muscle
46   nNOS expression and tNOx levels in skeletal muscle while these parameters did not change
47   in T2DM, perhaps due to higher insulin resistance (unchanged after intervention).

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Introduction

The World Health Organization (WHO) states that being overweight or obese is the fifth leading risk factor for global mortality (James 2008). Low-grade inflammation is a feature of both obesity and type 2 diabetes mellitus (T2DM) (Degens 2010; Krause et al. 2012a), which are characterized by a chronic pro-inflammatory state associated with an increased release of key pro-inflammatory cytokines, e.g. tumor necrosis factor alpha (TNF-α) from different sources (Degens 2010; Krause et al. 2012a). TNF-α signalling also induces activation of several pathways which initiate the production of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Newsholme and Krause 2012), which promote impairment of insulin signalling (Newsholme and Krause 2012).

Disrupted redox signalling or elevated oxidative stress (from prolonged periods of hyperglycaemia and/or elevated pro-inflammatory cytokines) is thought to underlie the vascular dysfunction observed in individuals with glucose intolerance and diabetes (Cersosimo and DeFronzo 2006). Likewise, it has been shown that individuals with T2DM have more pronounced systemic inflammation and oxidative stress than those with normal glucose tolerance, leading to decreased bioavailability of nitric oxide (a key mediator of vessel tone, glucose uptake and β-cell function) (Krause et al. 2012b; Krause et al. 2011; Newsholme et al. 2009; Newsholme et al. 2012).

Regular moderate intensity aerobic exercise is widely recommended for sedentary individuals due to its beneficial effect on insulin sensitivity and glycaemic control (Boule et al. 2001). It is also known that such exercise can improve glycaemic and lipid control in diabetes and consequently, reduce cardiovascular disease (CVD) risk factors through improvements in fasting blood lipids, postprandial glycaemia and lipaemia, blood pressure and body mass (Duncan 2006; Harding 2006). However, it is unclear which type, frequency

82 and intensity of exercise will be of most benefit for obese and/or T2DM individuals (Thomas

83 et al. 2006). Previous work has reported a reduced capacity for fat oxidation, a shift from fat

84 to carbohydrate utilisation at lower intensities during exercise and a lower exercise intensity

85 associated with the maximal rate of fat oxidation in people with T2DM relative to healthy

86 controls (Boon et al. 2007; Brun et al. 2007; Ghanassia et al. 2006). These findings have

87 contributed to the promotion of low intensity aerobic exercise training (at the intensity

88 corresponding to maximal rate of fat oxidation - "Fat-Max training") so as to increase fat

89 oxidation capacity (Brun et al. 2007), rather than moderate intensity training prescriptions

90 (corresponding to either 60-70% of VO_{2max} (Boon et al. 2007) or based on ventilatory

91 thresholds (Tvent; (Belli et al. 2007; Fujita et al. 1990). While fat oxidation may be elevated

92 under this intensity of exercise, information on the impact on other parameters of metabolic

93 status or oxidative stress in T2DM patients is lacking.

94   Therefore, the aim of this study was to investigate changes in nitric oxide availability

95 (and iNOS/nNOS expression), oxidative stress and inflammatory markers in response to

96 exercise training at two different intensities (Fat-Max *vs*. Tvent intensities) in obese males

97 with and without T2DM. Specifically, we analyzed cytokines and adipokines that are known

98 to modulate the availability of $NO^{\cdot}$ and also be involved in the development of insulin

99 resistance.

100

Research Design and Methods

Participants Characteristics

103   Twenty-five sedentary (not engaged in any regular physical activity for the last six

104 months) non-smoking male participants (52.8±7.2 years old), with body mass index (BMI)

105   >27 kg/m^2 volunteered for this study (12 obese controls *vs.* 13 obese T2DM, previously

106   diagnosed by their personal physicians). BMI ranged from 27.05 to 38.08 kg/m^2 for control

107   subjects and 27.3 to 37.5 kg/m^2 for T2DM. Informed consent was obtained from all

108   volunteers prior to the study. Research assessments and protocols were approved by the UCD

109   Dublin Human Research Ethics Committee. Participants were free from secondary

110   complications of diabetes at the time of recruitment.

111

Experimental Design

113   Participants were recruited via advertising in local newspapers and workplaces.

114   Participants attended the university laboratory for testing on five occasions. In session one,

115   body composition was measured and a submaximal incremental treadmill test was performed

116   to estimate aerobic capacity (VO$_{2max}$) and to measure ventilatory threshold. Total body

117   composition was assessed using dual energy X-ray absorptiometry (DEXA - Lunar iDXA,

118   GE Healthcare, Buckinghamshire, United Kingdom). In session two, performed one week

119   later, resting blood and skeletal muscle biopsy samples were taken following an overnight

120   fast. The participants then completed a six-minute constant load exercise bout at an intensity

121   corresponding to 35% estimated VO$_{2max}$, followed by a second six-minute bout at an intensity

122   corresponding to 25% estimated VO$_{2max}$. The format of session three which was performed

123   one week later was identical to session two, except for the intensity of the 2 six-minute

124   constant load exercise bouts which corresponded to 60% and 45% of estimated VO$_{2max}$

125   respectively. Participants were then randomly assigned to train (not supervised) at either low

126   (Fat-Max training) or moderate intensity (Tvent training), giving four groups: Obese Control

127   training at Fat-Max (Control Fat-Max); Obese Diabetic training at Fat-Max (T2DM Fat-

128   Max); Obese Control training at Tvent (Control Tvent) and Obese Diabetic training at Tvent

129   (Control Tvent).

130   The unsupervised training programme (16 weeks) consisted of a continuous outdoor

131   30 min walking, 3 times per week. Participants were provided with a heart rate (HR) monitor

132   (Polar RS400) with their individual zone of training pre-programmed. Midway (week 8)

133   through the training programme, participants returned to the laboratory (session four) and

134   completed both the incremental and four submaximal constant load exercise bouts, for

135   adjustment of their target HR training zone. Finally, after completing the 16 week

136   programme, participants attended the laboratory for a fifth testing session, during which

137   resting fasted blood and muscle samples were obtained and the exercise and body

138   composition tests were repeated. The experimental design is described in Figure 1.

139

140   **Estimated aerobic power (VO_{2max})**

141   Maximal oxygen consumption (VO_{2max}) was estimated from a sub-maximal

142   incremental walking test. Briefly, participants walked on a motor driven treadmill (Venus

143   200, HP Cosmos, Nussdorf-Traunstein, Germany) with a protocol of simultaneous changes in

144   speed and slope, designed to achieve a linear increase in workload (Porszasz et al. 2003).

145   The specific increments were calculated for each participant based on their age, body weight

146   and predicted aerobic capacity (Fujita et al. 1990). Expired gases were collected and analysed

147   using a breath by breath analyser (Quark B2, Cosmed, Rome, Italy). The test was terminated

148   when the subjects reached the 85% of their age-predicted maximal heart rate (220- age in

149   years). All participants were able to reach this point of exertion. VO_{2max} was then estimated

150   by linear regression of the VO_2 – heart rate relationship to the predicted maximal heart rate.

151 During the test, particular attention was paid to the VCO_2 and VE trends to verify that Tvent

152 was reached by every participant.

153

Sub-Maximum Constant Load Tests and Fat-Max determination

155   Each participant completed four 6-minute submaximal constant load treadmill

156 exercise bouts, at intensities corresponding to 25%, 35%, 45% and 60% of their estimated

157 VO_{2max}, as determined from the incremental test (Achten et al. 2002; Ghanassia et al. 2006).

158

Determination of training intensities

160   'Fat-max' training intensity: For each participant, the intensity corresponding to the

161 maximal rate of fat oxidation was calculated using the data obtained from the four

162 submaximal constant load bouts, as described previously (Ghanassia et al. 2006; Venables et

163 al. 2005). Briefly, data from the 5th and 6th minute of each bout were averaged and rate of

164 lipid oxidation calculated from VO_2 and VCO_2 (Peronnet and Massicotte 1991). This was

165 plotted against exercise intensity, and a third-order polynomial curve was fitted to the data

166 (Stisen et al. 2006). The intensity at the maximal point of the resultant curve was identified,

167 and the heart rate corresponding to this intensity was then used for prescription of the

168 participant's training zone. 'Tvent' training intensity: ventilatory threshold was identified

169 from the incremental exercise test, using the modified V-slope method (Sue et al. 1988). Two

170 experimenters calculated this point independently, and the average of the two VO_2 values was

171 used. Where the two VO_2 values differed by more than 100ml, a second method was used to

172 verify the VO_2 corresponding to Tvent (method of ventilatory equivalents; (Noonan and Dean

173 2000).

Biochemical analysis

Venous blood samples were obtained from an antecubital vein in heparin coated (for plasma acquisition) or clotting activator (for serum) Vacutainer^{TM} tubes using standard aseptic techniques. Samples were immediately centrifuged (at 4°C and 1000g for 15min), after which plasma or serum were removed and stored at −80°C for further analysis. Concentrations of glucose, total cholesterol and high-density lipoprotein cholesterol (HDL-C) were measured using a portable Cardiochek Analyser (Polymer Technology Systems, Indianapolis, Indiana, USA). Triglyceride (TG) concentration was quantified using a commercially available colorimetric assay (Tryglicerides Liquicolor, Wiebsbaden, Germany). Estimates of low-density lipoprotein cholesterol (LDL-C) concentration were calculated using the Friedewald formula (Friedewald et al. 1972). Glycosylated haemoglobin (% HbA1c) was assayed using DCA Vantage Serum Analyser (Siemens, Dublin, Ireland). Plasma glycerol was assessed using a colorimetric assay (Cayman Chemical Company, USA, Catalogue 10010755) according to manufacturer's instructions. Highly sensitive, enzyme-linked immunosorbent assay (EIA) methods were used to determine the concentration of leptin (Mercodia, Catalogue 10-1199-01, Uppsala, Sweeden) and adiponectin (Mercodia, Catalogue 10-1193-01) in plasma according to manufacturer's instructions. Quantification was made using a microplate reader (Molecular Devices SpectraMax Plus 384, Sunnyvale, California, United States). Serum high-sensitivity C-reactive protein (hsCRP) was assayed using a CRP high sensitivity assay kit (Cayman, Ireland). Blood was centrifuged in heparin-coated tubes and the red blood cell pellet homogenized in cold metaphosphoric acid. Redox state and glutathione metabolism was measured in erythrocytes by a modification of the 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB]/GSSG reductase recycling method, using the N-ethylmaleimide conjugating technique for GSSG sample preparation (Krause et al. 2007). Total antioxidant activity was measured using a colorimetric assay (Catalogue No: 709001,

199 Cayman, Ireland). Catalase activity was assessed using a Cayman Chemical Catalase Assay

200 Kit (Catalogue No: 707002) and oxidative damage in proteins by using the Cayman

201 Chemical's Protein Carbonyl Colorimetric Assay Kit (catalogue 10005020) (Fayh et al.

202 2012). Serum concentrations of IL-6 and TNF were quantified using a Human IL-6 and TNF-

203 alpha Quantikine ELISA Kit (Catalogue No: D6050 and DTA00C, R&D Systems, USA).

204 The choice between plasma or serum samples for the analysis was based on the assay

205 performed, technique sensitivity and kit manufacturer instructions. Serum was used for IL-6,

206 TNF-α and hsCRP; plasma for adiponectin, leptin, catalase, protein carbonyls, total

207 antioxidant activity, triglycerides and glycerol and whole blood for glucose, LDL, HDL,

208 HbA1c and total cholesterol measurements.

209

210 **Skeletal muscle microbiopsy and NOS protein expression**

211 Muscle biopsy samples were obtained using a spring-loaded and reusable instrument

212 (Magnum reusable core biopsy instrument MG1522; Bard, Dublin, Ireland). This technique

213 has been assessed in terms of patient tolerance and causes minimal or no discomfort (Hayot

214 et al. 2005). The muscle samples (~15 mg) were taken from the medial part of the vastus

215 lateralis muscle under local anaesthesia (1% lidocaine). The samples obtained from each

216 biopsy were immediately frozen in liquid nitrogen and stored at -80°C until required. After

217 removal from -80°C storage, tissue samples were thawed and homogenized in lysis buffer (20

218 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na_3VO_4,

219 10μg/ml Aprotinin, 10 μg/ml leupeptin, 3 mM benzamidine and 1 mM PMSF) using an

220 automated homogenizer (TissueLyser LT, Qiagen, Dublin Ireland) . The homogenate was

221 rotated at 4°C for 10 min, followed by centrifugation at 14,000g at 4°C for 10 min, after

222 which the supernatant was collected. Cellular protein concentration was determined using a

223   BCA protein Assay (Pierce, Rockford, IL, USA Catalogue No: 23225).   Protein samples

224   (15μg) were denatured in sample buffer and separated by 10% SDS PAGE. The proteins were

225   transferred onto a nitrocellulose membrane (Amersham Biosciences, Ireland), blocked in 5%

226   (BSA) and probed with the appropriate polyclonal antibodies; Anti-nNOS (1:10000 dilution,

227   Cell Signalling Technologies, USA), Anti-iNOS (1:10000 dilution, Cell Signalling

228   technologies, USA) and Anti-GAPDH (1:10000 dilution, Cell Signalling technologies, USA)

229   over night. Following overnight incubation, membranes were washed and incubated for 60

230   min at room temperature with horseradish peroxidase (HRP)-conjugated secondary

231   antibodies (1:30000 dilution, Cell Signalling technologies, USA). The blots were washed and

232   visualized with a horseradish peroxidase-based Supersignal West Pico chemiluminescent

233   substrate (Pierce). Results of digitalized images were expressed as mean ± S.D. using anti-

234   GAPDH as an expression control. The densities of the bands were quantified using ImageJ

235   version 1.44p (National Institutes of Health, USA).

236

237   **Nitric oxide product determinations (tNOx) in serum and skeletal muscle samples**

238   Frozen muscle samples were weighed, and homogenized in sterile PBS (phosphate

239   buffer saline, GIBCO, Ireland) using an automated homogenizer (TissueLyser LT, Qiagen,

240   Dublin Ireland). The homogenised tissue (and serum samples) were placed in an eppendorf

241   tube and centrifuged at 10,000g for 20minutes. The supernatant was ultra-centrifuged using

242   molecular weight cut-off filters (30kDa, Amicon, Millipore, Ireland) for 30 minutes at

243   100,000g. The supernatant was used for the remainder of the assays. Nitric oxide levels were

244   determined indirectly by quantification of their oxidised products of degradation, nitrates and

245   nitrites, using nitrate reductase and the Greiss reagent technique by a colorimetric kit

246 (Cayman Chemical Company, USA, Catalogue No: 780001). The inter-assay and intra-assay

247 coefficients of variation for the Greiss reaction were 5.6% and 3.6% respectively.

248

249 **Statistical Analysis**

250 Data are presented as mean and SD. Ryan-Joiner normality test was applied prior to

251 all analyses. For the following variables: glycaemia, HbA1c, insulin, HOMA-IR, HOMA-

252 beta, HDL, triglycerides, CRP, VO_{2max}, TNF-α, IL-6, total antioxidants and glycerol, which

253 were not-normally distributed, a logarithmic transformation was applied (Log_{10}). An

254 ANOVA General Linear Model (GLM) was performed to compare the four sub-groups with

255 three factors of interaction: 1) the presence or not of T2D (Diabetes vs. Control); 2) Time

256 (before and after exercise training); 3) exercise intensity (Fat-Max or Tvent). Post-hoc Tukey

257 test was further applied when appropriate. Finally, age, body fat percentage and estimated

258 VO_{2max} were added as co-variants in the ANOVA GLM. The alpha level was set at $P<0.05$.

259 Data were analysed using Minitab 16 software.

260

261 **Results**

262 **Adherence to Training**

263 After the completion of the training, the data from the HR monitors were downloaded

264 and individually analysed. Adherence to training was high (92%) with participants

265 completing 44±7 of the prescribed 48 walking sessions. In addition, all participants were

266 successful in training at the prescribed heart rate; the average difference between the

267 prescribed and recorded training heart rate was small (-0.5 bpm).

Comparison of the baseline blood biochemical variables between the obese control and the obese T2DM subjects

As expected, glycaemia (5.2±0.4 vs. 8.36±1.2 mmol/L) and HbA1C (5.6±0.1 vs. 7.3±0.9, % OR 38±1 mmol/mol vs. 56±2 mmol/mol) were higher in the T2DM group. Indicators of both insulin resistance (HOMA-IR) and beta-cell function (HOMA-β) were altered in T2DM, indicating that diabetic participants were insulin resistant and also may present an abnormal beta-cell function. Total cholesterol and LDL cholesterol (139.7±33.8 vs. 77.6±35.6 mg/dL) were lower in T2DM subjects. This was expected since the diabetic subjects were taking cholesterol-lowering statin drugs. VO_{2max} was lower in T2DM than obese controls (31.2±6.45 vs. 38.9±5.27 ml.kg^{-1}.min^{-1}, P<0.05). No differences were found between Control and T2DM for leptin, adiponectin, TNF-α and total antioxidants. IL-6 was significantly higher in the T2DM group, although body fat percentage as a co-variant affected this result. Significant differences were found in protein carbonyls between control and T2DM individuals (75.5±41.6 vs. 141.7±52.1 nmol/mL respectively). In addition, reduced glutathione (GSH) was lower in diabetic patients (32.7±4.4 vs. 21.8±2.8, nmol/mL).

Analysis of blood biochemistry parameters and body composition in response to exercise training

General metabolic variables such as glycaemia, lipid metabolism and HbA1c are shown in Table 1. No differences were found between pre- and post-training in any of the training groups. Similarly, no changes were observed in adiponectin, leptin or TNF-α (Table 2). With the exception of IL-6, the co-variants (% body fat, age and VO_{2max}) did not affect

290   any other variable. Body composition, VO_{2max} and blood pressure were also unchanged after

291   the interventions.

292

293   **Antioxidants and oxidative protein damage in response to exercise training**

294   As shown in Table 2, the major differences regarding oxidative stress markers and

295   antioxidants were related to the levels of protein carbonyls, being higher in T2DM subjects.

296   Catalase activity increased in diabetic subjects only after Tvent training intervention, but not

297   at Fat-Max (0.98 ± 0.22 at basal levels vs. 1.96 ± 0.3nmol/min/ml after Tvent training, Table 2).

298   The diabetic subjects responded to exercise training with increased protein oxidative damage

299   at both training intensities (Fat-Max and Tvent), as evidenced by decreased protein carbonyls,

300   particularly in the Tvent group (147.2 ± 52.6 at baseline and 66.57 ± 19.5nmol/ml after Tvent

301   training intervention). There were no changes in markers of oxidative stress or protein

302   damage in either control training group. No differences in glutathione metabolism were found

303   between pre- and post-training samples (Table 2). However, baseline values for GSH were

304   higher in the control group than in T2DM group and the difference remained after exercise

305   training at both intensities.

306

307   **Serum levels of nitric oxide metabolites in response to exercise training**

308   Regarding serum tNOx, in all groups and intensities, no significant differences were

309   found after the exercise training intervention (Table 2). On the other hand, baseline values for

310   tNOx were higher in the control group than in T2DM group (18.68 ± 2.78 vs.

311   12.34 ± 3.56µmol/L,) and this difference remained following exercise training at both

312   intensities.

Skeletal Muscle iNOS/nNOS Expression and nitric oxide metabolites levels in response to exercise training

We analysed the responses of iNOS/nNOS expression and the tNOx production in skeletal muscle samples to chronic (training) exercise (Figures 2A-D). Following training, the only significant change observed was an increase in the levels of nNOS and tNOx in the control group trained at Tvent intensity (Figure 2A). At basal levels, as expected, skeletal muscle nNOS was higher in controls than in diabetics and the opposite was found for iNOS expression levels, but this did not impact on the baseline tNOx levels between the groups (Figures 2A-D).

Discussion

The main finding of this study was that endurance exercise training, particularly at moderate intensity (Tvent), increased catalase activity and decreased protein oxidative damage in individuals who are obese with T2DM. However, this improvement was not accompanied by changes in glycaemia or body composition. These findings are in accordance with the observation that prescription of non-supervised physical activity alone does not improve glycaemic control in T2DM (Wisse et al. 2010). A further possible reason for not observing significant changes in variables such as HbA1c with exercise training is the regular use of anti-diabetic drugs which would improve many metabolic parameters and thus reduce the scope for further change. The exercise training program used in our intervention did not result in any detectable changes in nitric oxide production in obese T2DM subjects, although the levels of skeletal muscle nNOS expression and tNOx concentration were increased in the control obese group in response to moderate aerobic exercise training.

336   Structured exercise is considered an important cornerstone for achieving good

337   glycaemic control and reducing cardiovascular risk in type 2 diabetes (O'Hagan et al. 2013;

338   Praet and van Loon 2009) Several different protocols, intensities and types of exercise have

339   previously been promoted for the diabetic population (for review, read (O'Hagan et al. 2013;

340   Praet and van Loon 2009)). In the present study, we analysed the chronic effects of aerobic

341   training under two different intensities, which was performed three times per week, with

342   sessions lasting 30 minutes, for a period of 16 weeks.

343   It was decided to compare Fat-Max vs. Tvent intensities because it has previously

344   been suggested that low intensity protocols (which favour the use of fatty acids rather than

345   carbohydrates as a source of energy) might improve metabolic, inflammatory and oxidative

346   stress markers by inducing higher levels of fatty acid oxidation (Brun et al. 2007). This would

347   be beneficial for T2DM subjects, since the disorder is associated with an earlier shift from fat

348   to carbohydrate utilisation during exercise (Boon et al. 2007) and a reduced fat oxidative

349   capacity (16; 17). It has been reported previously that a single bout of low intensity exercise,

350   as opposed to high intensity exercise, substantially reduces the prevalence of hyperglycaemia

351   throughout the subsequent 24h post-exercise period in longstanding T2DM patients [35]. In

352   our hands, Fat-Max training did not change glycaemic or lipid profiles. Nevertheless, our

353   findings do not exclude possible beneficial effects of this type of training for T2DM subjects,

354   since we only evaluated fasting glycaemic values and not continuous daily variations, as

355   described by Manders et al [35]. Furthermore, while significant training effects were not

356   observed for most variables (glycaemia, lipid profile, HbA1c), calculation of Cohen's effect

357   sizes (results not shown) showed medium-to-large effects of training. Therefore it is possible

358   that including larger group sizes might have resulted in a significant training effect.

359   While NO· is an essential molecule for many physiological functions such as

360   skeletal muscle glucose uptake and vasomotricity (Newsholme et al. 2009), its availability is

361   decreased in diabetes. We aimed to analyse the nitric oxide production response of obese

362   T2DM to exercise training. Our results indicated that exercise at Fat-Max or Tvent

363   intensities, did not produce detectable change in NO• production within the T2DM groups.

364   However, the control group that trained at Tvent intensity responded by increasing the

365   production of tNOx and expression of nNOS. We have previously demonstrated that skeletal

366   muscle nNOS and iNOS expression levels are different between healthy obese, T2DM obese

367   and lean T2DM (Krause et al. 2012b). This may indicate that obesity, rather than diabetes

368   alone is the cause of the differential levels of tNOx intracellularly (muscle) and

369   extracellularly (serum). Increased circulating IL-6 may promote increased levels of iNOS

370   within the skeletal muscle of the diabetic subjects (Krause et al. 2012a). The fact that T2DM

371   subjects did not respond to the moderate intensity training (in contrast to controls) would

372   suggest that their lack of response may be caused by the insulin resistant state itself, since

373   cell-based insulin signalling is required for normal nNOS expression and nitric oxide

374   production (Krause et al. 2012b; Newsholme et al. 2009). Despite the lack of change in nitric

375   oxide production in our T2DM subjects, the possible beneficial effects of our exercise

376   protocol on the cardiovascular system of these patients cannot be excluded. For instance,

377   numerous interventions based on exercise and/or dietary changes have shown improved

378   vascular responses, such as an increase in blood flow without any significant changes to nitric

379   oxide production (Fayh et al. 2012; Monti et al. 2012). In addition, recent findings suggest

380   that vasodilatory prostanoids are important in determining endothelial response to Ach in

381   diabetic and non-diabetic subjects (Meeking et al. 2000). Thus increased prostaglandin-

382   mediated vasodilation may compensate for attenuated responses to NO·, as has been

383   previously reported in diabetic subjects (Meeking et al. 2000).

384   We have also confirmed that serum tNOx level (nitric oxide availability) is reduced

385   in patients with diabetes. We speculate that this response is due to the reduced availability of

386   circulating L-arginine (Newsholme et al. 2009; Newsholme et al. 2012). Decreased blood

387   levels of insulin, increased angiotensin II, hyper-homocysteinaemia, increased ADMA

388   (asymmetric ω-NG,NG-dimethylarginine), low plasma L-arginine and tetrahydrobiopterin

389   (BH4) are all conditions likely to decrease NO· production and which are also associated with

390   diabetes and cardiovascular disease (Newsholme et al. 2009). Recently, adipose tissue has

391   been implicated in the regulation of vascular function in humans via the release of vasoactive

392   cytokines called adipokines, including adiponectin and leptin (Antonopoulos et al. 2011). In

393   our hands, however, no differences were found in theses adipokines after the exercise

394   intervention and, for this reason, we cannot attribute the differences in nitric oxide to

395   alterations in adiponectin levels.

396   In insulin-resistant populations, several adipokines as well as muscle contraction-

397   induced factors, so-called myokines (i.e., IL-6), have been shown to modulate insulin

398   resistance and inflammatory status (Pedersen and Febbraio 2008). Although there is a

399   consensus that weight loss is associated with an increase in adiponectin and decreased levels

400   of leptin, TNF-α and high sensitivity C-reactive protein (hsCRP) (Madsen et al. 2008;

401   Monzillo et al. 2003), studies on medium-term effects of exercise without concomitant

402   weight loss are limited and produce somewhat inconsistent results (De Feyter et al. 2007;

403   Lambert et al. 2008; Stewart et al. 2007). Indeed, we did not find any changes in adipokines

404   (adiponectin and leptin), or cytokines (TNF-α) following training irrespective of exercise

405   intensity in any group, which may reflect the importance of weight loss and adiposity levels

406   on the low-grade inflammation found in obesity and diabetes. On the other hand, IL-6 was

407   higher in T2DM subjects. However, increments in IL-6 were correlated with greater body fat

408   content in this group compared with the non-diabetic group. Thus higher concentrations of

409   IL-6 in T2DM may be a consequence of greater body fat content, rather than diabetes itself.

410   Free radicals (i.e. ROS and $NO^{\cdot}$), are known to be involved in the pathogenesis of

411 diabetes (Brownlee 2001). Besides inhibiting intracellular insulin signalling, the

412 aforementioned metabolic disturbances in glucose and fat metabolism increase the formation

413 of Amadori-glycated proteins and advanced glycation end-products (AGE), which impair

414 receptor function for AGE (RAGE) and enhance ROS levels in almost all organ systems

415 (Brownlee 2001; Goldin et al. 2006). Chronic exposure to Amadori products, AGE and ROS

416 can cause vasculopathy, glomerulopathy (Brownlee 2001; Vincent et al. 2007) and

417 potentially also induce nerve cell damage (Vincent et al. 2007). In accordance, nutritional

418 (Vincent et al. 2007) and/or exercise interventions (Ji 2002) that modulate AGE, RAGE

419 and/or ROS formation have been reported to improve insulin sensitivity in experimental

420 rodent models. Despite the fact that our exercise intervention did not induce changes in

421 inflammatory profiles or body composition, T2DM patients who trained at Tvent improved

422 their antioxidant defences (catalase), resulting in lower oxidative damage, as represented by

423 the levels of protein carbonyls. On the other hand, glutathione metabolism did not change,

424 although at baseline levels, we demonstrated that diabetic subjects had lower levels of

425 reduced glutathione. This may suggest that production of this antioxidant, perhaps as a

426 consequence of decreased key amino acid availability for glutathione synthesis, was

427 compromised, as previously suggested (Newsholme et al. 2012).

428

429 **Conclusions and Perspectives**

430   In summary, moderate-intensity exercise may promote alternative health benefits

431 compared to exercise at Fat-Max intensities and, in the long term, may result in visible

432 changes in glycemic control and cardiovascular improvement resulting in fewer diabetic

433 complications. Obese, non-T2DM participants can respond to moderate intensity training by

434 increasing nNOS levels and tNOx levels in skeletal muscle cells, which may represent an

435 adaptive mechanism to exercise. However, NO related responses in T2DM patients did not

436 respond in the same positive direction as observed for the control group, which may be

437 attributable to their level of insulin resistance (unchanged after the exercise intervention). We

438 suggest that it is important to evaluate other types of exercise, including different intensities

439 and volumes of training in order to find the most appropriate exercise for obese and T2DM

440 population to improve cellular and metabolic function. Despite the limitations of this study

441 (absence of diet control and the small sample size), it is important to highlight that our

442 intervention, in spite of the low training volume intervention, induced positive modifications

443 among obese individuals with T2DM.

444

445 **Declaration of Interest**

446   The authors declare that there is no conflict of interest that could be perceived as

447 prejudicing the impartiality of the research reported.

448

449 **Authors Contribution Statement**

450   MK, JRK, COH, GDV, DS, and PM completed all sample collection. MK completed

451 all the biochemical and molecular analysis. JRK, COH and PM performed all body

452 composition and cardiorespiratory analysis. MK, GDV and PN co-wrote the manuscript. MK,

453 GDV, PN, CB, CM, DS, GD and COH provided experimental advice and helped with

454 manuscript revision. CM, GDV and PN were responsible for grant support with respect to

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Tables and Figures Legends

Table 1. Effects of exercise training on Subjects` Anthropometric, biochemical and

physiological characteristics.

Table 2. Effects of exercise training on Subjects` hormone, inflammatory and Oxidant/Anti-

oxidant profile.

Figure 1. Study design.

Figure 2. Representative Western blot of human skeletal muscle biopsies for nNOS/iNOS

and intracellular tNOx. **A**: in Obese Control Subjects training at 60% VO_{2max} intensity. **B**: in

Obese Control Subjects training at 35% VO_{2max} intensity. **C**: in T2DM Subjects training at

60% VO_{2max} intensity. **D:** in T2DM Subjects training at 35% VO_{2max} intensity. Levels of

nNOS and iNOS expression in the skeletal muscle of all subjects were measured. Total levels

nitrites and nitrates (tNOx) were measured as an indication of nitric oxide production within

the cells.   * $P<0.05$ when compared with baseline levels.