

The effects of aerobic exercise training at two different intensities in obesity and type 2 diabetes: implications for oxidative stress, low-grade inflammation and nitric oxide production

KRAUSE, Mauricio, RODRIGUES-KRAUSE, Josianne, O'HAGAN, Ciara, MEDLOW, Paul, DAVISON, Gareth, SUSTA, Davide, BOREHAM, Colin, NEWSHOLME, Philip, O'DONNELL, Mark, MURPHY, Colin and DE VITO, Giuseppe

Available from Sheffield Hallam University Research Archive (SHURA) at:

<http://shura.shu.ac.uk/8980/>

This document is the author deposited version. You are advised to consult the publisher's version if you wish to cite from it.

Published version

KRAUSE, Mauricio, RODRIGUES-KRAUSE, Josianne, O'HAGAN, Ciara, MEDLOW, Paul, DAVISON, Gareth, SUSTA, Davide, BOREHAM, Colin, NEWSHOLME, Philip, O'DONNELL, Mark, MURPHY, Colin and DE VITO, Giuseppe (2013). The effects of aerobic exercise training at two different intensities in obesity and type 2 diabetes: implications for oxidative stress, low-grade inflammation and nitric oxide production. *European Journal of Applied Physiology*, 114 (2), 251-260.

Copyright and re-use policy

See <http://shura.shu.ac.uk/information.html>

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

4
5 **Authors:** Mauricio Krause^{1, 2*}; Josianne Rodrigues-Krause¹; Ciara O'Hagan^{2,3,}; Paul
6 Medlow⁴; Gareth Davison; Davide Susta⁵; Colin Boreham²; Philip Newsholme⁶; Mark
7 O'Donnell⁷; Colin Murphy¹ and Giuseppe De_Vito^{2,*}

8
9 **Authors Affiliations:** ¹Biomedical Research Group, Department of Science, Institute of
10 Technology Tallaght, Dublin, Ireland; ²Institute for Sport and Health, University College
11 Dublin, Dublin, Ireland; ³Academy of Sport and Physical Activity, Sheffield Hallam
12 University, Sheffield, United Kingdom; ⁴Sport and Exercise Sciences Research Institute,
13 University of Ulster, Jordanstown, Co Antrim, Northern Ireland, UK; ⁵Dublin City
14 University, Dublin, Ireland and ⁶School of Biomedical Sciences, CHIRI Biosciences and
15 Faculty of Health Sciences, Curtin University, Perth, Western Australia 6845; ⁷Department of
16 Vascular and Endovascular Surgery, Royal Victoria Hospital, Belfast, Northern Ireland, UK.

17 **Short Title:** *Exercise training responses in T2DM: Low vs. Moderate intensities*

18 **Keywords:** diabetes, obesity, adipokines, oxidative stress, nitric oxide, blood, skeletal muscle

19 **List of Abbreviations:** GSH (Reduced Glutathione); GSSG (Glutathione Disulfide); tNOx
20 (nitric oxide metabolites); T2DM (type 2 diabetes mellitus); CRP (C-reactive protein).

21 **Abstract word count:** 250 **Main text word count:** 4454 **N° references:** 49

22 **Number of Figures:** 2 **Number of Tables:** 2

23
24 **Correspondence Address:** Mauricio Krause, Institute of Technology Tallaght, Tallaght,
25 Dublin 24, Dublin-Ireland and University College Dublin, Dublin 4, Dublin-Ireland. **E-mail**
26 **Address:** mauriciokrause@hotmail.com

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/m²) 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).

48

49

50

51

52

53

54

55

56

57

58 **Introduction**

59 The World Health Organization (WHO) states that being overweight or obese is the
60 fifth leading risk factor for global mortality (James 2008). Low-grade inflammation is a
61 feature of both obesity and type 2 diabetes mellitus (T2DM) (Degens 2010; Krause et al.
62 2012a), which are characterized by a chronic pro-inflammatory state associated with an
63 increased release of key pro-inflammatory cytokines, e.g. tumor necrosis factor alpha (TNF-
64 α) from different sources (Degens 2010; Krause et al. 2012a). TNF- α signalling also induces
65 activation of several pathways which initiate the production of free radicals, reactive oxygen
66 species (ROS) and reactive nitrogen species (RNS) (Newsholme and Krause 2012), which
67 promote impairment of insulin signalling (Newsholme and Krause 2012).

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

76 Regular moderate intensity aerobic exercise is widely recommended for sedentary
77 individuals due to its beneficial effect on insulin sensitivity and glycaemic control (Boule et
78 al. 2001). It is also known that such exercise can improve glycaemic and lipid control in
79 diabetes and consequently, reduce cardiovascular disease (CVD) risk factors through
80 improvements in fasting blood lipids, postprandial glycaemia and lipaemia, blood pressure
81 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

101 **Research Design and Methods**

102 **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/m² 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/m² for control
107 subjects and 27.3 to 37.5 kg/m² 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

112 **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 ($220 - \text{age}$ in
149 years). All participants were able to reach this point of exertion. VO_{2max} was then estimated
150 by linear regression of the $VO_2 - \text{heart rate}$ 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 T_{vent}
152 was reached by every participant.

153

154 **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

159 **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. ‘ T_{vent} ’ 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 T_{vent} (method of ventilatory equivalents; (Noonan and Dean
173 2000).

174 **Biochemical analysis**

175 Venous blood samples were obtained from an antecubital vein in heparin coated (for
176 plasma acquisition) or clotting activator (for serum) VacutainerTM tubes using standard
177 aseptic techniques. Samples were immediately centrifuged (at 4°C and 1000g for 15min),
178 after which plasma or serum were removed and stored at -80°C for further analysis.
179 Concentrations of glucose, total cholesterol and high-density lipoprotein cholesterol (HDL-C)
180 were measured using a portable Cardiochek Analyser (Polymer Technology Systems,
181 Indianapolis, Indiana, USA). Triglyceride (TG) concentration was quantified using a
182 commercially available colorimetric assay (Tryglicerides Liquicolor, Wiebsbaden, Germany).
183 Estimates of low-density lipoprotein cholesterol (LDL-C) concentration were calculated
184 using the Friedewald formula (Friedewald et al. 1972). Glycosylated haemoglobin (%
185 HbA1c) was assayed using DCA Vantage Serum Analyser (Siemens, Dublin, Ireland).
186 Plasma glycerol was assessed using a colorimetric assay (Cayman Chemical Company, USA,
187 Catalogue 10010755) according to manufacturer's instructions. Highly sensitive, enzyme-
188 linked immunosorbent assay (EIA) methods were used to determine the concentration of
189 leptin (Mercodia, Catalogue 10-1199-01, Uppsala, Sweden) and adiponectin (Mercodia,
190 Catalogue 10-1193-01) in plasma according to manufacturer's instructions. Quantification
191 was made using a microplate reader (Molecular Devices SpectraMax Plus 384, Sunnyvale,
192 California, United States). Serum high-sensitivity C-reactive protein (hsCRP) was assayed
193 using a CRP high sensitivity assay kit (Cayman, Ireland). Blood was centrifuged in heparin-
194 coated tubes and the red blood cell pellet homogenized in cold metaphosphoric acid. Redox
195 state and glutathione metabolism was measured in erythrocytes by a modification of the 5,5'-
196 dithiobis(2-nitrobenzoic acid) [DTNB]/GSSG reductase recycling method, using the N-
197 ethylmaleimide conjugating technique for GSSG sample preparation (Krause et al. 2007).
198 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 Na₃VO₄,
219 10 μ g/ml Aprotinin, 10 μ g/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 \pm 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).

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

270 As expected, glycaemia (5.2 ± 0.4 vs. 8.36 ± 1.2 mmol/L) and HbA1C (5.6 ± 0.1 vs.
271 7.3 ± 0.9 , % OR 38 ± 1 mmol/mol vs. 56 ± 2 mmol/mol) were higher in the T2DM group.
272 Indicators of both insulin resistance (HOMA-IR) and beta-cell function (HOMA- β) were
273 altered in T2DM, indicating that diabetic participants were insulin resistant and also may
274 present an abnormal beta-cell function. Total cholesterol and LDL cholesterol (139.7 ± 33.8
275 vs. 77.6 ± 35.6 mg/dL) were lower in T2DM subjects. This was expected since the diabetic
276 subjects were taking cholesterol-lowering statin drugs. VO_{2max} was lower in T2DM than
277 obese controls (31.2 ± 6.45 vs. 38.9 ± 5.27 ml.kg⁻¹.min⁻¹, $P<0.05$). No differences were found
278 between Control and T2DM for leptin, adiponectin, TNF- α and total antioxidants. IL-6 was
279 significantly higher in the T2DM group, although body fat percentage as a co-variant affected
280 this result. Significant differences were found in protein carbonyls between control and
281 T2DM individuals (75.5 ± 41.6 vs. 141.7 ± 52.1 nmol/mL respectively). In addition, reduced
282 glutathione (GSH) was lower in diabetic patients (32.7 ± 4.4 vs. 21.8 ± 2.8 , nmol/mL).

283

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

286 General metabolic variables such as glycaemia, lipid metabolism and HbA1c are
287 shown in Table 1. No differences were found between pre- and post-training in any of the
288 training groups. Similarly, no changes were observed in adiponectin, leptin or TNF- α (Table
289 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 ± 0.3 nmol/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 ± 19.5 nmol/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 ± 3.56 μ mol/L,) and this difference remained following exercise training at both
312 intensities.

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

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

322

323 **Discussion**

324 The main finding of this study was that endurance exercise training, particularly at
325 moderate intensity (Tvent), increased catalase activity and decreased protein oxidative
326 damage in individuals who are obese with T2DM. However, this improvement was not
327 accompanied by changes in glycaemia or body composition. These findings are in accordance
328 with the observation that prescription of non-supervised physical activity alone does not
329 improve glycaemic control in T2DM (Wisse et al. 2010). A further possible reason for not
330 observing significant changes in variables such as HbA1c with exercise training is the regular
331 use of anti-diabetic drugs which would improve many metabolic parameters and thus reduce
332 the scope for further change. The exercise training program used in our intervention did not
333 result in any detectable changes in nitric oxide production in obese T2DM subjects, although
334 the levels of skeletal muscle nNOS expression and tNO_x concentration were increased in the
335 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 \cdot 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 \cdot , 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 these 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[•]), 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 adaptative 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
455 TSR: Strand III – Core Research Strengths Enhancement (Ireland).

456

457 **Funding:** This work was supported by the Institute of Technology Technological Sector
458 Research (TSR): Strand III – Core Research Strengths Enhancement (Ireland) and the Irish
459 Research Council for Science, Engineering and Technology.

460

461 REFERENCES

- 462 Achten J, Gleeson M, Jeukendrup AE (2002) Determination of the exercise intensity that elicits
463 maximal fat oxidation. *Med Sci Sports Exerc* 34: 92-97
- 464 Antonopoulos AS, Lee R, Margaritis M, Antoniadou C (2011) Adiponectin as a regulator of vascular
465 redox state: therapeutic implications. *Recent Pat Cardiovasc Drug Discov* 6: 78-88
- 466 Belli T, Ackermann MA, Ribeiro LF, Langeani R, Galdino da Silva R, Baldissera V (2007) Lactate and
467 ventilatory thresholds in type 2 diabetic women. *Diabetes Res Clin Pract* 76: 18-23
- 468 Boon H, Blaak EE, Saris WH, Keizer HA, Wagenmakers AJ, van Loon LJ (2007) Substrate source
469 utilisation in long-term diagnosed type 2 diabetes patients at rest, and during exercise and
470 subsequent recovery. *Diabetologia* 50: 103-112
- 471 Boule NG, Haddad E, Kenny GP, Wells GA, Sigal RJ (2001) Effects of exercise on glycemic control and
472 body mass in type 2 diabetes mellitus: a meta-analysis of controlled clinical trials. *JAMA* 286: 1218-
473 1227
- 474 Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:
475 813-820
- 476 Brun JF, Jean E, Ghanassia E, Flavier S, Mercier J (2007) Metabolic training: new paradigms of
477 exercise training for metabolic diseases with exercise calorimetry targeting individuals. *Ann Readapt*
478 *Med Phys* 50: 528-534, 520-527
- 479 Cersosimo E, DeFronzo RA (2006) Insulin resistance and endothelial dysfunction: the road map to
480 cardiovascular diseases. *Diabetes Metab Res Rev* 22: 423-436
- 481 De Feyter HM, Praet SF, van den Broek NM, Kuipers H, Stehouwer CD, Nicolay K, Prompers JJ, van
482 Loon LJ (2007) Exercise training improves glycemic control in long-standing insulin-treated type 2
483 diabetic patients. *Diabetes Care* 30: 2511-2513
- 484 Degens H (2010) The role of systemic inflammation in age-related muscle weakness and wasting.
485 *Scand J Med Sci Sports* 20: 28-38
- 486 Duncan GE (2006) Exercise, fitness and cardiovascular disease risk in type 2 diabetes and the
487 metabolic syndrome. *Curr Diab Re* 6: 29-35
- 488 Fayh AP, Krause M, Rodrigues-Krause J, Ribeiro JL, Ribeiro JP, Friedman R, Moreira JC, Reischak-
489 Oliveira A (2012) Effects of L-arginine supplementation on blood flow, oxidative stress status and
490 exercise responses in young adults with uncomplicated type I diabetes. *Eur J Nutr*
- 491 Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density
492 lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18: 499-
493 502
- 494 Fujita Y, Kawaji K, Kanamori A, Matoba K, Yajima Y, Takeuchi A, Ishii K (1990) Relationship between
495 age-adjusted heart rate and anaerobic threshold in estimating exercise intensity in diabetics.
496 *Diabetes Res Clin Pract* 8: 69-74
- 497 Ghanassia E, Brun JF, Fedou C, Raynaud E, Mercier J (2006) Substrate oxidation during exercise: type
498 2 diabetes is associated with a decrease in lipid oxidation and an earlier shift towards carbohydrate
499 utilization. *Diabetes Metab* 32: 604-610
- 500 Goldin A, Beckman JA, Schmidt AM, Creager MA (2006) Advanced glycation end products: sparking
501 the development of diabetic vascular injury. *Circulation* 114: 597-605

502 Harding AHG, S.J. and Wareham, N.J. (2006) Population impact of strategies for identifying groups at
503 risk of type 2 diabetes. *Prev Med* 42: 364-368

504 Hayot M, Michaud A, Koechlin C, Caron MA, Leblanc P, Prefaut C, Maltais F (2005) Skeletal muscle
505 microbiopsy: a validation study of a minimally invasive technique. *Eur Respir J* 25: 431-440

506 James WP (2008) WHO recognition of the global obesity epidemic. *Int J Obes (Lond)* 32 Suppl 7:
507 S120-126

508 Ji LL (2002) Exercise-induced modulation of antioxidant defense. *Ann N Y Acad Sci* 959: 82-92

509 Krause M, Bittencourt A, Homem de Bittencourt PI, McClenaghan NH, Flatt PR, Murphy C,
510 Newsholme P (2012a) Physiologic concentrations of IL-6 directly promote insulin secretion, signal
511 transduction, nitric oxide release and redox status in a clonal pancreatic beta-cell line and mouse
512 islets. *J Endocrinol*

513 Krause M, Rodrigues-Krause J, O'Hagan C, De Vito G, Boreham C, Susta D, Newsholme P, Murphy C
514 (2012b) Differential Nitric Oxide levels in the blood and skeletal muscle of Type 2 diabetic subjects
515 may be consequence of adiposity: a preliminary study. *Metabolism*

516 Krause MS, McClenaghan NH, Flatt PR, de Bittencourt PI, Murphy C, Newsholme P (2011) L-arginine
517 is essential for pancreatic beta-cell functional integrity, metabolism and defense from inflammatory
518 challenge. *J Endocrinol* 211: 87-97

519 Krause MS, Oliveira LP, Jr., Silveira EM, Vianna DR, Rossato JS, Almeida BS, Rodrigues MF, Fernandes
520 AJ, Costa JA, Curi R, de Bittencourt PI, Jr. (2007) MRP1/GS-X pump ATPase expression: is this the
521 explanation for the cytoprotection of the heart against oxidative stress-induced redox imbalance in
522 comparison to skeletal muscle cells? *Cell Biochem Funct* 25: 23-32

523 Lambert CP, Wright NR, Finck BN, Villareal DT (2008) Exercise but not diet-induced weight loss
524 decreases skeletal muscle inflammatory gene expression in frail obese elderly persons. *J Appl Physiol*
525 105: 473-478

526 Madsen EL, Rissanen A, Bruun JM, Skogstrand K, Tonstad S, Hougaard DM, Richelsen B (2008)
527 Weight loss larger than 10% is needed for general improvement of levels of circulating adiponectin
528 and markers of inflammation in obese subjects: a 3-year weight loss study. *Eur J Endocrinol* 158:
529 179-187

530 Meeking DR, Browne DL, Allard S, Munday J, Chowienyczyck PJ, Shaw KM, Cummings MH (2000)
531 Effects of cyclo-oxygenase inhibition on vasodilatory response to acetylcholine in patients with type
532 1 diabetes and nondiabetic subjects. *Diabetes Care* 23: 1840-1843

533 Monti LD, Setola E, Lucotti PC, Marrocco-Trischitta MM, Comola M, Galluccio E, Poggi A, Mammi S,
534 Catapano AL, Comi G, Chiesa R, Bosi E, Piatti PM (2012) Effect of a long-term oral l-arginine
535 supplementation on glucose metabolism: a randomized, double-blind, placebo-controlled trial.
536 *Diabetes Obes Metab*

537 Monzillo LU, Hamdy O, Horton ES, Ledbury S, Mullooly C, Jarema C, Porter S, O'valle K, Moussa A,
538 Mantzoros CS (2003) Effect of lifestyle modification on adipokine levels in obese subjects with insulin
539 resistance. *Obes Res* 11: 1048-1054

540 Newsholme P, Homem De Bittencourt PI, C OH, De Vito G, Murphy C, Krause MS (2009) Exercise and
541 possible molecular mechanisms of protection from vascular disease and diabetes: the central role of
542 ROS and nitric oxide. *Clin Sci (Lond)* 118: 341-349

543 Newsholme P, Krause M (2012) Nutritional Regulation of Insulin Secretion: Implications for Diabetes.
544 *Clinical Biochemist Reviews* 33: 35-47

545 Newsholme P, Rebelato E, Abdulkader F, Krause M, Carpinelli A, Curi R (2012) Reactive oxygen and
546 nitrogen species generation, antioxidant defenses, and beta-cell function: a critical role for amino
547 acids. *J Endocrinol* 214: 11-20

548 Noonan V, Dean E (2000) Submaximal exercise testing: clinical application and interpretation. *Phys
549 Ther* 80: 782-807

550 O'Hagan C, De Vito G, Boreham CA (2013) Exercise prescription in the treatment of type 2 diabetes
551 mellitus : current practices, existing guidelines and future directions. *Sports Med* 43: 39-49

552 Pedersen BK, Febbraio MA (2008) Muscle as an endocrine organ: focus on muscle-derived
553 interleukin-6. *Physiol Rev* 88: 1379-1406
554 Peronnet F, Massicotte D (1991) Table of nonprotein respiratory quotient: an update. *Can J Sport Sci*
555 16: 23-29
556 Porszasz J, Casaburi R, Somfay A, Woodhouse LJ, Whipp BJ (2003) A treadmill ramp protocol using
557 simultaneous changes in speed and grade. *Med Sci Sports Exerc* 35: 1596-1603
558 Praet SF, van Loon LJ (2009) Exercise therapy in type 2 diabetes. *Acta Diabetol* 46: 263-278
559 Stewart LK, Flynn MG, Campbell WW, Craig BA, Robinson JP, Timmerman KL, McFarlin BK, Coen PM,
560 Talbert E (2007) The influence of exercise training on inflammatory cytokines and C-reactive protein.
561 *Med Sci Sports Exerc* 39: 1714-1719
562 Stisen AB, Stougaard O, Langfort J, Helge JW, Sahlin K, Madsen K (2006) Maximal fat oxidation rates
563 in endurance trained and untrained women. *Eur J Appl Physiol* 98: 497-506
564 Sue DY, Wasserman K, Moricca RB, Casaburi R (1988) Metabolic acidosis during exercise in patients
565 with chronic obstructive pulmonary disease. Use of the V-slope method for anaerobic threshold
566 determination. *Chest* 94: 931-938
567 Thomas DE, Elliott EJ, Naughton GA (2006) Exercise for type 2 diabetes mellitus. *Cochrane Database*
568 *Syst Rev*: CD002968
569 Venables MC, Achten J, Jeukendrup AE (2005) Determinants of fat oxidation during exercise in
570 healthy men and women: a cross-sectional study. *J Appl Physiol* 98: 160-167
571 Vincent AM, Perrone L, Sullivan KA, Backus C, Sastry AM, Lastoskie C, Feldman EL (2007) Receptor for
572 advanced glycation end products activation injures primary sensory neurons via oxidative stress.
573 *Endocrinology* 148: 548-558
574 Wisse W, Rookhuizen MB, de Kruif MD, van Rossum J, Jordans I, ten Cate H, van Loon LJ, Meesters
575 EW (2010) Prescription of physical activity is not sufficient to change sedentary behavior and
576 improve glycemic control in type 2 diabetes patients. *Diabetes Res Clin Pract* 88: e10-13
577
578
579
580
581
582
583
584
585
586
587

588

589

590

591

592

593

594

595 **Tables and Figures Legends**

596

597 **Table 1.** Effects of exercise training on Subjects` Anthropometric, biochemical and
598 physiological characteristics.

599

600 **Table 2.** Effects of exercise training on Subjects` hormone, inflammatory and Oxidant/Anti-
601 oxidant profile.

602

603 **Figure 1.** Study design.

604

605 **Figure 2.** Representative Western blot of human skeletal muscle biopsies for nNOS/iNOS
606 and intracellular tNOx. **A:** in Obese Control Subjects training at 60% VO_{2max} intensity. **B:** in
607 Obese Control Subjects training at 35% VO_{2max} intensity. **C:** in T2DM Subjects training at
608 60% VO_{2max} intensity. **D:** in T2DM Subjects training at 35% VO_{2max} intensity. Levels of
609 nNOS and iNOS expression in the skeletal muscle of all subjects were measured. Total levels
610 nitrites and nitrates (tNOx) were measured as an indication of nitric oxide production within
611 the cells. * $P < 0.05$ when compared with baseline levels.

612

613

614

615