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Implication of free fatty acids in thrombin generation and fibrinolysis in vascular inflammation in aged Zucker rats and evolution with aging

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Summary. *Background:* The metabolic syndrome (MetS) and aging are associated with modifications in blood coagulation factors, vascular inflammation, and increased risk of thrombosis. *Objectives:* Our aim was to determine concomitant changes in thrombin generation in the blood compartment and at the surface of vascular smooth muscle cells (VSMCs) and its interplay with adipokines, free fatty acids and metalloproteinases (MMPs) in obese Zucker rats that share features of the human MetS. *Methods:* Obese and age-matched lean Zucker rats were compared at 25 and 80 weeks of age. Thrombin generation was assessed by calibrated automated thrombography (CAT). *Results:* Endogenous thrombin potential (ETP) was increased in obese rats independent of platelets and age. Clot half-lysis time was delayed with obesity and age. Interleukin (IL)-1β and IL-13 were increased with obesity and age respectively. Addition of exogenous fibrinogen, leptin, linoleic or palmitic acid increased thrombin generation in plasma whereas adiponectin had an opposite effect. ETP was increased at the surface of VSMCs from obese rats and addition of exogenous palmitic acid further enhanced ETP values. Gelatinase activity was increased in aorta at both ages in obese rats and MMP-2 activity was increased in VSMCs from obese rats. *Conclusions:* Our study demonstrated in MetS an early prothrombotic phenotype of the blood compartment reinforced by procoagulant properties of dedifferentiated and inflammatory VSMCs. Mechanisms involved (1) increased fibrinogen and impaired fibrinolysis and (2) increased saturated fatty acids responsible for additive procoagulant effects. Whether specifically targeting this hypercoagulability using direct thrombin inhibitors would improve outcome in MetS is worth investigating.

Keywords: vascular aging, blood coagulation test, obesity, fatty acids, thrombin generation.

67 **Introduction**

68 Atherothrombotic events and venous thromboembolism are associated with the metabolic
69 syndrome (MetS), a cluster of risk factors for cardiovascular disease including insulin
70 resistance (IR), abdominal adiposity, dyslipidemia, and hypertension (Dandona et al., 2005).
71 Likewise, obesity is causally related to the high prevalence of MetS. Inflammation in MetS
72 results in endothelial dysfunction and increased arterial stiffness (Weiss et al., 2013), probably
73 through the action of matrix metalloproteinases (MMPs) (Halcox et al., 2009). Aging is also
74 associated with intimal thickening, breaks in the internal elastic lamina and impaired
75 endothelial function leading to increased arterial stiffness (Wang et al., 1996).

76 A further cascade of obesity-induced chronic inflammation leads to increased tissue factor
77 (TF) (Samad et al., 2001) through the NF-κB pathway (Sonnenberg et al., 2004). Von
78 Willebrand factor (VWF) participates in the prothrombotic state found in MetS (Lim et al.,
79 2004). Total thrombin generation and platelet reactivity are increased in type 2 diabetes and
80 older obese women (Beijers et al., 2010). Furthermore, as far as fibrinolysis is concerned,
81 chronic inflammation, abdominal obesity, and IR all increase plasminogen activator inhibitor-
82 1 (PAI-1) production, so reducing plasminogen conversion and leading to a hypofibrinolytic
83 state (Alessi and Juhan-Vague, 2008; Suehiro et al., 2012).

84 Adipokine levels (adiponectin, leptin) as well as free fatty acid (FFA) metabolism are
85 changed significantly in MetS (Matsuzawa et al., 2004; Wakil and Abu-Elheiga, 2009). Both
86 are known also to be directly or indirectly implicated in haemostasis and increased thrombosis
87 (Konstantinides et al., 2001; Restituto et al., 2010). Since haemostasis is modified in the MetS
88 and during aging our hypothesis is that MetS, the related adipokines, and FFAs have a major
89 impact on haemostasis changes, increased thrombotic risk and worsen the vascular phenotype.
90 A major challenge is to elucidate the mechanisms leading to increased thrombosis during
91 MetS and in the natural course of aging, and how they are related to the interaction between

92 blood haemostasis and the vascular wall. Rodent models that mimic human MetS are major
93 tools for understanding this pathophysiology (Sloboda et al., 2012).

94 Obese Zucker rats have a missense point mutation (fa/fa) in the leptin receptor gene that leads
95 to hyperphagia and marked obesity (Phillips et al., 1996). These rats display also many other
96 aspects of the human condition, such as IR, hypertension, and increased plasma lipid levels.
97 We have shown previously that obese Zucker rats exhibited an increased age-dependent
98 arterial stiffening which was greater in obese than lean, as well as endothelial dysfunction
99 with increased systemic oxidative stress (Sloboda et al., 2012).

100 We have developed therefore a strategy combining “adult” (25-week-old) and “old” (80-
101 week-old) Zucker rats with MetS characteristics and their lean controls and a vascular smooth
102 muscle cell (VSMC) approach to investigate the role of FFAs and vascular inflammation in
103 the prothrombotic properties of MetS. We first explored thrombin generation and its
104 functional consequences on the fibrin network and on fibrinolysis in the blood compartment.
105 To get insights into the underlying mechanisms we then examined thrombin generation at the
106 surface of Zucker rat VSMCs and their MMP activity. We demonstrated that obesity from at
107 least 25 weeks triggers increased thrombin generation in the blood compartment and at the
108 surface of VSMCs via increased FFAs and associated vascular inflammation.

109

110 **Materials and methods**

111 ***Animals***

112 Male Zucker rats with the MetS (MSZR, fa/fa; *n*=18) and their age-matched male lean
113 Zucker rat controls (LZR, FA/-; *n*=18) were obtained from the breeding colony (animal
114 facility, Faculty of Medicine, University of Lorraine, France). The animals were maintained
115 at a constant temperature of 22-24°C, with a 12h light-dark cycle (light beginning at 8 AM)
116 and given free access to water and standard chow (A04, Scientific Animal Food and

117 Engineering advance, Augy, France). The metabolic status of MSZR and LZR has been
118 published previously (Sloboda et al., 2012).
119 Eighty weeks of age corresponds to 5 weeks before the mean maximum life span of rats from
120 our local breeding colony.
121 This study was carried out in accordance with recommendations of the Animal Ethics
122 Committee of the Institut National de la Santé et de la Recherche Médicale and conformed to
123 the Guide for the Care and Use of Laboratory Animals, published by the National Institutes
124 of Health. The protocols were approved by the Animal Ethics Committee of the Institut
125 National de la Santé et de la Recherche Médicale.
126
127 ***Blood sampling***
128 Rats were anesthetized with isoflurane and whole blood was collected via a carotid catheter
129 into syringes containing one-tenth the volume of 0.106 M sodium citrate. Platelet count was
130 determined with an automatic cell counter (Micros 60 ABX model, Montpellier, France).
131 Blood was centrifuged at 190*g* for 10 min at room temperature to obtain platelet-rich plasma
132 (PRP) and then at 1750*g* for 10 min to obtain platelet-poor plasma. PRP was adjusted to
133 200×10^9 platelets/l by addition of autologous platelet-poor plasma and used for platelet
134 aggregation and thrombin generation. Platelet-free plasma (PFP) was obtained by
135 centrifugation of platelet-poor plasma at 13000*g* for 30 min at 4°C, and frozen at –80°C.
136
137 ***Preparation of Arterial Cryo-Sections***
138 Artery cryo-sections were collected in the cross-sectional orientation and used subsequently
139 for *in situ* gelatin zymography. The descending thoracic aorta was embedded in Optimal
140 Cutting Temperature (OCT) medium and frozen using iso-pentane pre-cooled in liquid N_2 and

141 stored at -80ºC until cryo-sectioning. Cryo-sections were cut at a thickness of 5 µm and
142 mounted onto glass slides (Leica, Milton Keynes, UK) and stored at -80 ºC until use.
143

144 ***Cell culture***

145 The descending thoracic aorta was excised from rats after isoflurane anaesthesia (4.5% in
146 1.5 l/min dioxygen) and exsanguination. VSMCs were isolated as described previously (Ait
147 Aissa et al., 2015). VSMCs were grown in DMEM/F12 supplemented with 10% foetal bovine
148 serum (Lonza, Basel, Switzerland). For thrombin generation assays, VSMCs at passages 3-5
149 were seeded (7500 cells/well) in 96-well tissue culture flat-bottom plates
150 (MICROTEST™96), grown to subconfluence and washed with HBS before use.
151

152 ***Platelet aggregation***

153 Blood was centrifuged at 190*g* for 4 min followed by 70 seconds at 1900*g* at room
154 temperature to obtain PRP and then platelets were sedimented by centrifugation at 5000*g* for
155 4 min. Platelets were re-suspended in Tyrode buffer (5 mM Hepes, 137 mM NaCl, 2.7 mM
156 KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, pH
157 7.3). Platelet aggregation was measured by turbidimetry at 37°C under stirred conditions. PRP
158 or washed platelets were adjusted to 200×10^9 platelets/l and were stimulated by 5 µg/ml
159 collagen or 5 µM ADP (SD Innovation, Frouard, France). Aggregation was followed for 10
160 min using a TA-8V aggregometer (SD Innovation).
161

162 ***Thrombin generation assay***

163 Calibrated automated thrombinography (CAT) in PRP or PFP was performed in a microtiter
164 plate fluorometer (Fluoroskan Ascent, ThermoLabsystems, Helsinki, Finland) using a
165 dedicated software program (Thrombinoscope BV, Maastricht, The Netherlands) as reported
166 previously (Regnault et al., 2004). All reagents were used at half the ordinary volume as

167 follows: 40 µl PRP or PFP, 10 µl of 5 pM recombinant human tissue factor (TF) (Dade
168 Behring, Marburg, Germany) and phospholipid vesicles (PV) consisted of
169 phosphatidylcholine-serine-ethanolamine (PC/PS/PE) 60/20/20 mole% at a final
170 concentration of 4 µM equivalent PS, 10 µl fluorogenic substrate and calcium. PV were
171 replaced by buffer in PRP and VSMC experiments. Round-bottom 96-well Greiner blue
172 plates were used for PFP and PRP, and MICROTEST™96 plates for VSMC monolayers.
173 Thrombin generation curves were recorded in triplicate. Thrombin generation was monitored
174 also following supplementing PFP with adiponectin or leptin (BioVision, San Francisco,
175 USA), with fibrinogen (Sigma-Aldrich, St Louis, USA), or with palmitic acid or linoleic acid
176 (Sigma-Aldrich).
177
178 ***Coagulation and circulating parameters***
179 Prothrombin and FVIII were measured in PFP samples diluted 1:40-80 in factor diluent
180 (Instrumentation Laboratory, Le Pré Saint Gervais, France). For each assay 50 µl of diluted
181 sample were added to 50 µl of human prothrombin-deficient plasma (Siemens Healthcare
182 Diagnostics SAS, Saint-Denis, France) or FVIII deficient plasma (Dade Behring, Deerfield,
183 USA). After 1 min of incubation at 37°C in a KC10 coagulometer, coagulation was started by
184 addition of 80 µl of Thromborel® S. Calibration curves were generated using the reference
185 plasma Unicalibrator (Diagnostica Stago, Asnières, France). Fibrinogen was measured in PFP
186 samples diluted 1:10-20 in Owren-Koller buffer (Diagnostica Stago, Asnières, France).
187 Unicalibrator was used to generate calibration curves. After 4 min of incubation at 37°C in a
188 KC10 coagulometer, coagulation was started by addition of 100 µl of Fibriquik (Biomérieux-
189 Trinity Biotech, Bray, Ireland). Antithrombin levels were measured with the Coamatic®
190 antithrombin test kit from Chromogenix, and TAT with the Enzygnost® TAT micro
191 (Instumentation Laboratory). TF and TF pathway inhibitor (TFPI) activities were measured in
192 PFP using the Actichrome® tissue factor and Actichrome® TFPI activity assay respectively

193 (American Diagnostica, Stamford, CT). PAI-1 levels were measured with the rat PAI-1 total
194 antigen ELISA kit from Innovative Research, Inc. IL-13 and IL-1β concentrations were
195 measured with the IL13 and IL-1 beta rat ELISA kits from Invitrogen. MMP-9 levels were
196 measured with the Quantikine rat total MMP-9 immunoassay from R&D Systems. VCAM-1
197 was assessed with the rat VCAM-1 ELISA kit from Elabscience.
198
199 ***In vitro fibrinolytic test***
200 PFP (20 µl) was diluted by addition of 40 µl buffer containing 5 pM recombinant TF, PV at 4
201 µM equivalent PS, 5 nM rabbit thrombomodulin (TM) (American Diagnostica, Greenwich,
202 USA) and 4 µg/ml recombinant human tissue Plasminogen Activator (tPA) Actilyse®
203 (Boehringer Ingelheim, Ingelheim am Rhein, Germany). Clot formation was initiated by
204 addition of 10 µl of 100 mM $CaCl_2$. To monitor clot lysis, absorbance was read kinetically at
205 405 nm using a microplate reader. To standardize the figure, for each sample basal optical
206 density (OD) after lysis was subtracted from each point of the curve. Half lysis time was
207 defined as the time required to reach half-maximal variation in OD.
208
209 ***Microscopy of fibrin fiber ultrastructure***
210 The thrombin generation assay was performed in order to generate fibrin for fixation using the
211 same TF and PV concentrations as in the CAT experiments. This was done using plasma on
212 paper disks and a Rhodamine substrate was used (Ninivaggi et al., 2012). Immediately after
213 thrombin generation was finished (50 min for each run), the mineral oil was removed from the
214 well and a solution of glutaraldehyde (grade I) in phosphate buffered saline (PBS) (Sorensen's
215 PBS, pH 7.2) was applied. This was put at room temperature for 1h and then kept at 4°C
216 overnight. The samples were then washed 5 times with PBS and a secondary fixation was
217 performed in OsO_4 (1%) in sodium cacodylate (200 nM, pH 7.4) for 1h at RT. The samples
218 were then dehydrated with increasing concentrations of ethanol each during 3 min (30%,

219 50%, 70%, 90%, 100%) and the last step (100%) was performed 3 times. Further dehydration
220 was accomplished by a hexamethyldisilazane (HMDS)/ethanol solution (1:1) for 3 min and
221 HMDS for 10 min. The samples were removed from the wells and left to dry. In order to
222 visualize the samples with a Phenom G2Pro scanning electron microscopy (SEM) (Phenom-
223 World, Eindhoven, the Netherlands), they were put on stubs using carbon tabs and coated
224 with gold.
225 For each sample, 3 to 5 pictures were analyzed. Fiber thickness was measured using ImageJ
226 software (version 1.48v). For each picture 100 measurements were performed. The density of
227 the fibers was calculated from the pictures by counting the number of fibers that crossed a line
228 of 26.8 μm (Konings et al., 2011).
229

230 ***Rat cytokine antibody array***

231 The Rat Cytokine Array Panel A (Cat # ARY008) from R&D system (Minneapolis, MN) was
232 used to probe cytokines in PFP from MSZR and LZR by following the procedures
233 recommended by the manufacturer. Bound antibodies were detected by chemiluminescence
234 using the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica,
235 MA). This was performed once with a plasma pool from 5 to 6 animals to reduce inter-animal
236 variability in each group.
237

238 ***Phospholipid Procoagulant Activity***

239 The chromogenic assay measuring the phospholipid-related procoagulant activity (PPA) in
240 VSMCs was performed as described previously for plasma (Membre et al., 2008;
241 Wagenvoord et al., 1994). VSMCs cultured in 96 well plates were washed and 50 μl of 50
242 mM Tris, 175 mM NaCl, pH 7.9 (TBS) containing 2 g/l bovine serum albumin (BSA) were
243 added as well as 50 μl of activated factor X (1.2 nM), activated factor V (2.4 nM), $CaCl_2$ (15
244 mM) and 50 μl of bovine prothrombin (6 μM) plus Z-Gly-Gly-Arg-AMC substrate (1.25 mM)

245 in 20 mM HEPES pH 7.5 containing 60 g/l BSA. The plate was placed in the Fluoroskan
246 Ascent fluorometer and allowed to warm up to 37°C for 5 min before kinetic readings were
247 taken over 10 min. Phospholipid concentration was estimated from the initial rate of thrombin
248 formation by reference to a standard curve constructed with PV, and expressed as PS
249 equivalents.
250
251 ***Western blot***
252 Cell extracts were obtained by lysing VSMCs in complete Lysis-M buffer (Roche Diagnostics
253 Corporation, Basel, Switzerland). Detergent-soluble fractions were retained, and protein
254 concentrations in samples were determined using a Bradford protein assay (Bio-Rad, Hercule,
255 USA). Lysates containing 30 µg of protein were electrophoresed on polyacrylamide gels (8%
256 gel), transferred to Hybond-C nitrocellulose membranes (transblot turbo, Bio-Rad, Hercule,
257 USA) and blotted with the following antibodies: α-smooth muscle actin (αSMA), 4/1000
258 (Sigma-Aldrich), smooth muscle myosin heavy chain (SM-MHC), 1/1000 (Abcam;
259 Cambridge, UK); smoothelin, 1/500 (Santa Cruz Biotechnology, USA); integrin α_v, 1/1000
260 (Santa Cruz Biotechnology, Dallas Texas); integrin β_3 1/500 (Merck Millipore, Billerica,
261 USA) and tubulin, 2/1000 (Sigma-Aldrich). After rinsing, incubation with a secondary rabbit
262 antibody 1/1000 (α_v, β_3, smoothelin, SM-MHC, Sigma-Aldrich) and mouse antibody 1/1000
263 (αSMA, tubulin, Sigma-Aldrich). Reactions were visualised by the ECL Western Blot
264 Detection Kit (Bio-Rad, Hercule, USA) after incubation with peroxidase conjugates 1/2000
265 (GE Healthcare, Little Chalfont, UK). Tubulin was used as loading control and the protein
266 expression was normalized to tubulin.
267
268 ***In situ gelatin zymography***
269 *In situ* gelatin zymography was performed to determine the gelatinase activity across the
270 aortic wall using DQ-gelatin (Life Technologies, Paisley, UK) as described previously (Mook

271 et al., 2003). Fluorescein isothiocyanate (FITC, 1/110), and 4',6-diamidino-2-phenylindole
272 (DAPI, 1/150) filters were used to visualize the degree of gelatinase activity and the
273 localisation of nuclear tissue by fluorescence microscopy using a x20 optical objective
274 (Keynece, Osaka, Japan). Analysis of average fluorescence was performed for three 20 µm
275 thick profile lines across 3 arterial wall regions for each sample.
276
277 ***Zymography analysis***
278 VSMCs from LZR or MSZR (passage 4-6) were seeded (50 000 cells/well) in 6-well culture
279 plates in DMEM/F-12 supplemented with 10% foetal bovine serum (life technology Thermo
280 Fisher Scientific, Waltham, USA). Cells were grown to subconfluence and after 16h in serum-
281 free medium, cells were washed with PBS (Sigma-Aldrich), the medium was changed and
282 cells were incubated for 4h, 8h or 20h at 37°C. Conditioned media were then removed and
283 centrifuged at 500*g* for 10 min at room temperature and used for the determination of MMP-2
284 secretion.
285 Conditioned media were analyzed for gelatin degradation by electrophoresis under non-
286 reducing conditions on a 10% polyacrylamide-SDS gel containing 0.1% gelatin. Gels were
287 washed for 1h at room temperature in a 2% triton X-100 solution and incubated overnight at
288 37°C in 50 mM Tris–HCl/10 mM $CaCl_2$ (pH 7.6) buffer.
289 Gels were stained in a 0.1% coomassie Blue (G250)/45% methanol/10% acetic acid solution
290 and de-stained in a 10% acetic acid/20% methanol solution. White lysis strips, indicative of
291 gelatinolytic activity, were revealed and scanned (Fujifilm LAS 4000, Life sciences,
292 Branford, USA). Densitometric analysis was made using MultiGauge software (Fuji, Tokyo,
293 Japan). Foetal bovine serum diluted at 1% in serum free medium was used as a positive
294 control.
295
296 ***Statistical analysis***

Results are presented as mean ± standard error of the mean. Data were analyzed by a one-way or two-way ANOVA, followed by a Fisher's test for multiple comparisons to evaluate the influence of age and strain and their interaction on the different variables. In the case of SEM measurements, the differences in fiber thickness were analyzed using the Mann Whitney U test.

Results

Platelet aggregation, thrombin generation and fibrinolysis were all impaired with the MetS and/or aging.

Platelet count in blood was increased in MSZR at both ages compared to the same aged LZR (**Table 1**). Platelet aggregation using washed platelets and collagen as a strong agonist was not significantly modified as shown by the mean maximum aggregation (**Figure 1A**). For platelet aggregation in PRP using ADP, mean maximum aggregation was increased in 80 week-old MSZR and LZR compared to 25 week-old controls (**Figure 1B**). The F1+2 fragment was analysed to evaluate the *in vivo* reactivity of the coagulation system. The amount of F1+2 fragment was increased in 25 week-old MSZR compared to the same aged LZR (**Table 1**). Thrombin generation measurement was performed as an integrative *in vitro* phenotype of coagulation. Adult and very old MSZR had a significantly increased endogenous thrombin potential (ETP) compared to same aged LZR. The other thrombin generation parameters (lag time, peak, and velocity) were not changed significantly except for the time to peak which was increased in obese at both ages (**Table 1; Figure. 1C**). The ratio of thrombin generation in PFP and PRP compared to 25 week-old LZR was made to evaluate the platelet reactivity impact on thrombin generation. Interestingly, thrombin generation was more increased in PRP from MSZR at 25 week of age compared to 80 week-old rats (**Figure. 1D).** The coagulation parameters, TF, TFPI, prothrombin and fibrinogen, were all increased in

MSZR compared to LZR at both ages. TFPI was decreased and fibrinogen was increased with age in MSZR and prothrombin was increased with age in LZR. FVIII was increased significantly with age and MetS in 80 week-old MSZR. Antithrombin measurements showed no modification in MSZR and LZR rats (**Table 1**). Fibrin clots were characterised by SEM. Computerised analysis of the SEM images showed a decrease of fibrin fiber thickness in MSZR compared to LZR at both ages while fiber density was only increased in 80 week-old LZR (**Figure. 1E-G**). Circulating levels of PAI-1 were increased in both 80 week-old LZR and MSZR (**Figure. 1H**). In a fibrinolysis test (**Figure. 1I**), half-time lysis was increased in MSZR compared to LZR at both ages and aging significantly increased half-time lysis in both groups (**Figure. 1J**). Maximal lysis speed was not modified (**Figure. 1K**).

Inflammation, metabolic factors and free fatty acids modified thrombin generation.

Fibrinogen concentration was correlated highly to ETP (r = 0.069) and supplementing plasma with exogenous fibrinogen at concentrations that agreed with the changes between MSZR and LZR gradually increased ETP (**Figure. 2A-B**). The 1.2-fold increase in ETP with the 2.5 mg/mL concentration is consistent with the 1.4 increase in plasma fibrinogen in MSZR. We have then tested the effects of addition of exogenous leptin, adiponectin, linoleic acid and palmitic acid to PFP at concentrations selected to encompass the range previously reported for each molecule in MSZR. (Sloboda et al., 2012; Godin et al., 2013). Addition of leptin or adiponectin elicited similar concentration-dependent changes in ETP whatever the group of rat. The two adipokines had opposite effects on thrombin generation, leptin increased ETP whereas adiponectin decreased it (**Figure. 2C-D**). The two lower concentrations of added linoleic acid (0.75 and 1.5 mg/mL) had clear procoagulant effects whereas the higher concentration (3 mg/mL) was less effective in increasing thrombin generation (**Figure. 2E-F**). There was a significant increase in thrombin generation for all added concentrations of

palmitic acid whatever the group of rat. The results show an additive effect of FFAs on MSZR plasma.

Plasma cytokines were increased both with MetS and aging.

To explore inflammation in our model we performed a plasma cytokine array of 27 cytokines in order to provide qualitative data that will subsequently be used to quantify cytokines known likely to promote prothrombotic phenotypes (**Figure. 3**). Panel A presents pictures of the cytokine array membranes. A 50% variation between two groups was chosen as a threshold to classify cytokines into 4 groups. The first group of 5 cytokines showed no modifications (**Figure. 3B**), a second group of 8 cytokines were increased with MetS (**Figure. 3C**), a third group of 3 cytokines were increased with aging (**Figure. 3D**) and a last group of 11 cytokines were increased with both MetS and aging (**Figure. 3E**). The highest variation between 25 week-old MSZR and LZR was found for IL-1β (> 3000 % variation) and the highest variation between 80 week-old and 25 week-old rats was observed for IL-13 (> 400 % variation). ELISAs performed with individual rat PFP for IL-1β and IL-13 showed an increase of these cytokine levels in LZR and MSZR with age (**Figure. 3F, G**). IL-13 was increased also in 80 week-old MSZR compared to same aged LZR.

MetS and aging-induced inflammation and haemostasis impairment were related to alteration of VSMCs.

To explore the contribution of VSMCs, thrombin generation was measured at the surface of cultured VSMCs isolated from LZR and MSZR. Thrombin generation with PFP from LZR and MSZR was always increased at the surface of MSZR VSMCs compared to LZR VSMCs. Remarkably, addition of palmitic acid in LZR VSMCs increased thrombin generation to the level of MSZR independently of the PFP used (**Figure 4A**). MSZR VSMCs displayed increased procoagulant phospholipids at their surface compared to LZR VSMCs (**Figure 4B**).

374 Integrin subunit α_v was increased in MSZR compared to LSZ VSMCs while the β_3 subunit
375 was not modified. VSMC differentiation markers α-SMA, SM-MHC and smoothelin,
376 interestingly, were all decreased in MSRZ VSMCs compared to LZR VSMCs (**Figure 4C-D**).
377 Thus, *in situ* gelatin zymography was performed to explore MMP activity through gelatinase
378 activity (**Figure 4E**). Figure 4E shows representative photographs of *in situ* gelatin
379 zymography in aorta, gelatinase activity is in green. Mean gelatinase activity in the aortic wall
380 was increased in 25 and 80 week-old MSZR compared to age matched LZR aortas (**Figure**
381 **4F**). However, age did not modulate gelatinase activity. At the cellular level MSZR VSMCs
382 displayed increased MMP-2 secretion compared to LZR VSMCs (**Figure 4G-H**). Circulating
383 levels of MMP-9 were increased in 80 week-old MSZR whereas VCAM-1 was increased in
384 25 week-old MSZR compared to same aged LZR and in 80 week-old LZR (**Figure I J**).

385

386 **Discussion**

387 The aim of the present study was to determine concomitant changes in the haemostasis system
388 and VSMC phenotype and their interplay with FFAs and MMPs during aging in obese rats
389 compared to lean rats of the same age. Our results demonstrated (1) increased thrombin
390 generation in MetS in plasma as early as 25 weeks of age, independently of platelets and at
391 the surface of VSMCs; (2) reinforcement of this hypercoagulability by reduced plasma
392 fibrinolysis; (3) no influence of aging on plasma thrombin generation; (4) an age-related
393 increase in platelet aggregation and clot half lysis time and, (5) contribution of saturated FFAs
394 to the increased thrombin generation both in plasma and at the surface of VSMCs.
395 Increased thrombotic risk can be attributed to three factors: abnormalities in the vessel wall, in
396 blood flow, and in haemostasis including coagulation and fibrinolysis. We found previously
397 that MSZR presented endothelial dysfunction as shown by increased circulating VWF. This

398 endothelial dysfunction was exacerbated during aging as shown by increases in both VWF
399 and soluble CD146 (Sloboda et al., 2012).
400 Few studies have used Zucker rats to look at haemostasis and to our knowledge none have
401 been performed in very old Zucker rats. Paul *et al* found that 12 week-old diabetic Zucker rats
402 presented unmodified *in vitro* platelet reactivity (Paul et al., 2007). Recently Shang *et al* have
403 shown increased thrombosis, increased thrombin generation and decreased fibrinolysis in 7 to
404 10 week-old diabetic Zucker rats (Shang et al., 2014). They found also decreased platelet
405 reactivity to collagen and ADP in obese rats in PRP. In PRP, we found increased platelet
406 aggregation using ADP in 80 week-old MSZR and LZR rats compared to 25 week-old
407 controls, but not between rats of the same age. In addition, we were not able to aggregate
408 platelets using collagen. Washed platelets were able to aggregate when triggered with
409 collagen but we did not find any significant changes with obesity or with age. These changes
410 might be related to the metabolic differences existing between rats since they used diabetic
411 Zucker rats while we used obese Zucker rats that only develop diabetes very late with age.
412 Moreover, platelet count was not modified in the diabetic Zucker rats of the Shang *et al* study
413 while we found a 25% increased count in MSZR compared to LZR at both ages. Interestingly,
414 platelet-related thrombin generation showed a very important increase in 25 week-old MSZR
415 compared to thrombin generation made with PFP. Altogether, increased platelet aggregation
416 to ADP with age concomitant to increased platelet count in obese Zucker rats is in favor of a
417 prothrombotic state.
418 To better assess the prothrombotic state in obese and aged rats we investigated *in vivo*
419 thrombin generation by measuring F1+2 fragments, which were increased in MSZR
420 indicating increased *in vivo* formation of thrombin with MetS. As expected, MetS also
421 increased the *in vitro* thrombin generation capacity of plasma, but this ability was not
422 modified with age. This change in the *in vitro* reactivity of the coagulation system points out
423 the role of several components including metabolic factors and the vascular wall. Regarding

424 individual clotting factors it was clear that TF increased in MSZR as well as its inhibitor
425 (TFPI). Increased prothrombin concentration leads to higher thrombin generation and can
426 contribute to the increased ETP in MSZR. Other procoagulant factors such as FVII, FVIII and
427 VWF are known to be increased with MetS and aging. Metabolic factors such as leptin and
428 adiponectin can participate in haemostasis. Leptin has been suggested previously to represent
429 a link between obesity and atherothrombosis (Petrini et al., 2016). It has been reported that
430 leptin enhanced platelet aggregation while adiponectin reduced it (Konstantinides et al., 2001;
431 Restituto et al., 2010). Adiponectin has been involved also in the endothelium anticoagulation
432 function (Lee et al., 2011) since it increased endothelial TFPI synthesis (Chen et al., 2008).
433 We found in all Zucker rats a strong positive correlation between plasma TPFI and
434 adiponectin concentrations dosed previously (data not shown) (Sloboda et al., 2012).
435 Moreover, in our study, we found for the first time that leptin increased ETP and that
436 adiponectin decreased it. Despite it being a modest effect, it argues for a major involvement of
437 adipokines in the regulation of thrombin generation.
438 Fibrinogen concentration was correlated also to ETP and we confirmed that increased plasma
439 fibrinogen increased ETP (Kumar et al., 1994). Thrombin linked to fibrin can possibly be
440 protected from inhibition by antithrombin, in the same way as it is protected from inhibition
441 when bound to TM (Bourin, 1987). This may participate in explaining the increased time to
442 peak observed in MSZR and increased ETP with no significantly increased peak.
443 We found that fibrinogen concentration was increased in MSZR and during aging. In favor of
444 the relevance of this result it has been shown that synthesis of fibrinogen is upregulated by
445 inflammatory cytokines such as IL-6 (Morozumi et al., 2009). The consequence of an
446 increased thrombin generation was an increased fibrin network formation in MSZR as shown
447 by thinner fibrin fibers (Wolberg, 2007). The increase in PAI-1 with aging in LZR as well as
448 in MSZR is relevant to human physiology since it is known that during aging PAI-1 is
449 associated with an increased thrombotic risk. In addition, the fibrinogen concentration

450 increased during aging but the mechanisms underlying this association with thrombotic risk
451 are unclear (Cesari et al., 2010). Human fibrinolysis is also impaired in the MetS with a
452 decrease in clot lysis ability linked to increased PAI-1 (Pandolfi et al., 2001). Organization of
453 the fibrin network is likely due to the increased thrombin generation found in MSZR
454 (Wolberg, 2007). Moreover, clots with thinner fibrin fibers are more resistant to lysis than
455 clots with thick fibers (Gabriel et al., 1992). This is supported by the increased half-time lysis
456 found in MSZR and very old Zucker rats. Other factors must be implicated since fiber
457 thickness was unchanged with age in both groups whereas fibrinolysis time increased only
458 during aging indicating the formation of a denser clot. In line with this, adiponectin may act
459 as an anticoagulant molecule. Indeed, full length adiponectin reduces platelet aggregation,
460 inhibits TF and enhances TFPI expression at the surface of endothelial cells (Chen et al.,
461 2008; Restituto et al., 2010). Both adiponectin and IL-13 increase the expression of MMPs
462 which can degrade fibrinogen (Firszt et al., 2014; Hotary et al., 2002; Wanninger et al., 2011).
463 Consistent with this, we found an increase in IL-13 plasmatic concentration with aging and
464 also with the MetS in 80 week-old MSZR which presents the same variations as plasma levels
465 of MMP-9 and FVIII. Whether adiponectin interplays directly with fibrinogen remains an
466 open question. The increase in FVIII with MetS and associated inflammatory stimuli was
467 anticipated in Zucker rats as it is in humans (Begbie et al., 2000; Kotronen et al., 2011).
468 Inflammation during aging and in the MetS triggers vascular remodelling. Fibrinogen
469 (Lominadze et al., 2010) as well as fibrin and fibrin degradation products have
470 proinflammatory functions that can modify VSMC phenotype (Lu et al., 2011). Cytokines in
471 the plasma, as shown in the array presented here, are increased by the MetS, aging, or both.
472 Our data indicated that the more relevant proinflammatory cytokines such as IL-1α, IL-1β, IL-
473 2, IL-3, and IL-6 were increased early with the MetS while few anti-inflammatory cytokines
474 were increased with MetS and aging (IL-10, IL-1ra, IL-17). Our cytokine array made with a
475 pool of plasma for each group was checked using ELISA measurements with individual

476 samples for the two main cytokines involved in the regulation of haemostasis (IL-13 and IL-
477 1β). IL-13 changes were confirmed while IL-1β increased only with aging but not with the
478 MetS at 25 weeks of age. This points to a determinant role of age in complex vascular
479 pathologies including several comorbidities. IL-1β has a pleiotropic effect in the development
480 of atherothrombosis through its action on leukocyte adhesion to the vascular wall and
481 induction of procoagulant activity (Dinarello, 2011; Libby et al., 1986). Recently, inhibition
482 of IL-1β and subsequent reduction of inflammation (without modification of lipid levels) in
483 patients with previous episodes of myocardial infarction was found to reduce recurrent
484 cardiovascular events (Ridker et al., 2017). These findings are in line with the increase of
485 circulating IL-1β and increased activity of haemostasis with age we observed in MSZR.
486 Therefore, exploration of haemostasis function in MSZR with inhibition of IL-1β could be of
487 interest.
488 Other factors related to MetS that can potentiate the modifications we observed in MSZR
489 haemostasis are FFAs. Saturated FFAs such as palmitic acid are known to be associated with
490 ischemic heart disease and increase postprandial concentrations of fibrinogen (Pacheco et al.,
491 2006; Simon et al., 1995). One other mechanism proposed recently to explain the
492 thrombogenic effect of palmitic acid was its ability to induce extracellular release of histones
493 (Shrestha et al., 2013). Histones are known to promote thrombin generation through platelet
494 activation (Semeraro et al., 2011). Additionally, palmitic acid was measured recently in
495 diabetic Zucker rats pointing out a 2.75 times increased concentration in obese rats (0.68 g/l in
496 LZR vs 1.87 g/l in MSZR) (Godin et al., 2013). A similar increase was observed for a
497 polyunsaturated FFA, linoleic acid. We supplemented 25 week-old LZR PFP with linoleic or
498 palmitic acid to reach MSZR plasma concentrations. We showed for the first time a direct
499 effect of FFAs on thrombin generation confirming the prothrombotic effect of palmitic acid.
500 All these FFAs, pro-inflammatory cytokines and coagulation factors can have deleterious
501 effects on the vascular wall. We have shown previously the presence of endothelial

502 dysfunction in MSZR (Sloboda et al., 2012). In the present study we studied VSMCs in more
503 detail. Interestingly, thrombin generation measured at the surface of VSMCs from MSZR was
504 increased compared to LZR VSMCs. This increase can be related to the increased
505 procoagulant phospholipids at the surface of MSZR VSMCs. We showed recently that
506 thrombin generation at the surface of VSMC from spontaneously hypertensive rats (SHR)
507 leads to increased ETP and VSMCs were responsible for a prothrombotic phenotype in SHR
508 rats. In the same way as for SHR rats, increased VSMC-supported thrombin generation can be
509 a mechanism implicated in the prothrombotic phenotype we have observed in Zucker rats. In
510 these cellular experiments addition of palmitic acid exacerbated also thrombin generation over
511 MSZR VSMCs.
512 MMPs are related to FFAs, obesity-related diseases such as type 2 diabetes and overall,
513 inflammation. In our model, mean gelatinase activity, focusing on MMP-2 and -9 activities,
514 was increased in MSZR. These molecules are responsible for the degradation of type IV
515 collagen, elastin, fibronectin and laminin, among other proteins. It is known that FFAs and
516 insulin lead to hyperactivity of MMP-2 and -9 (Boden et al., 2008). The close relation
517 between MMPs and insulin was demonstrated also in Zucker rats (Zhou et al., 2005). IL-13
518 was increased in old rats and is known to be an activator of MMPs (Firszt et al., 2014). This
519 increase in aortic MMP activity in the intima with aging has been described in rats and was
520 two-fold higher in old versus young nonhuman primates (Li et al., 1999; Wang et al., 2007).
521 In addition, MMP activity may participate also in age-related vascular remodeling in the
522 aortic media since MMPs accumulate around elastic fibers in the aortic media (Li et al.,
523 1999), which become fragmented with age-associated increases in arterial stiffness which thus
524 increases cardiovascular risk. Interestingly, MMP production can be stimulated through
525 integrin $\alpha_v\beta_3$ (Bendeck et al., 2000). Concerning this pathway, we found an increase of the α_v
526 subunit in MSZR VSMCs and MMP-2 secretion was increased in MSZR compared to LZR.
527 Moreover, we have shown previously that this integrin is responsible for thrombin generation

528 supported by VSMCs and it argues for its role in vascular remodeling (Mao et al., 2012). Very
529 interestingly all VSMC differentiation markers we tested were downregulated in MSZR and
530 even absent concerning SM-MHC. This illustrates a phenotype switch from contractile to
531 secreting VSMCs occurring in vascular diseases such as atherosclerosis (Lacolley et al.,
532 2012).
533 In conclusion, our study demonstrates in MetS a prothrombotic phenotype of the blood
534 compartment reinforced by procoagulant properties of the vascular wall. Regarding the
535 mechanisms, fibrinogen contributes to this hypercoagulable phenotype in plasma at an early
536 stage of MetS. Leptin and adiponectin exert moderate opposite effects on thrombin generation
537 precluding a major contribution of adipokines. An increase in proinflammatory cytokines
538 likely increased MMP activity inducing a VSMC dedifferentiated phenotype exhibiting
539 procoagulant properties. An increase in FFAs contributes to the increased thrombin
540 generation both in plasma and at the surface of VSMCs. Plasma from MSZR and palmitic
541 acid elicit additive procoagulant effects. The potential benefit of direct thrombin inhibitors
542 should be investigated both on haemostatic balance in blood compartments and on the cellular
543 phenotypic modulation within the vessel wall, and MMP production in MetS and its
544 complications with aging.
545

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References

Ait Aissa, K., Lagrange, J., Mohamadi, A., Louis, H., Houppert, B., Challande, P., Wahl, D., Lacolley, P., and Regnault, V. (2015). Vascular smooth muscle cells are responsible for a prothrombotic phenotype of spontaneously hypertensive rat arteries. Arterioscler. Thromb. Vasc. Biol. *35*, 930–937.

Alessi, M.-C., and Juhan-Vague, I. (2008). Metabolic syndrome, haemostasis and thrombosis. Thromb. Haemost. *99*, 995–1000.

Begbie, M., Notley, C., Tinlin, S., Sawyer, L., and Lillicrap, D. (2000). The Factor VIII acute phase response requires the participation of NFkappaB and C/EBP. Thromb. Haemost. *84*, 216–222.

Beijers, H.J.B.H., Ferreira, I., Spronk, H.M.H., Bravenboer, B., Dekker, J.M., Nijpels, G., ten Cate, H., and Stehouwer, C.D.A. (2010). Body composition as determinant of thrombin generation in plasma: the Hoorn study. Arterioscler. Thromb. Vasc. Biol. *30*, 2639–2647.

Bendeck, M.P., Irvin, C., Reidy, M., Smith, L., Mulholland, D., Horton, M., and Giachelli, C.M. (2000). Smooth muscle cell matrix metalloproteinase production is stimulated via alpha(v)beta(3) integrin. Arterioscler. Thromb. Vasc. Biol. *20*, 1467–1472.

Boden, G., Song, W., Pashko, L., and Kresge, K. (2008). In vivo effects of insulin and free fatty acids on matrix metalloproteinases in rat aorta. Diabetes *57*, 476–483.

Bourin, M.C. (1987). [Effect of rabbit thrombomodulin on the inhibition of thrombin by the antithrombin-heparin complex: role of the acid domain of thrombomodulin]. Comptes Rendus Académie Sci. Sér. III Sci. Vie *304*, 173–176.

Cesari, M., Pahor, M., and Incalzi, R.A. (2010). Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. Cardiovasc. Ther. *28*, e72–e91.

Chen, Y.-J., Zhang, L.-Q., Wang, G.-P., Zeng, H., Lü, B., Shen, X.-L., Jiang, Z.-P., and Chen, F.-P. (2008). Adiponectin inhibits tissue factor expression and enhances tissue factor pathway inhibitor expression in human endothelial cells. Thromb. Haemost. *100*, 291–300.

Dandona, P., Aljada, A., Chaudhuri, A., Mohanty, P., and Garg, R. (2005). Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. Circulation *111*, 1448–1454.

Dinarello, C.A. (2011). Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood *117*, 3720–3732.

Dinarello, C.A., Simon, A., and van der Meer, J.W.M. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat. Rev. Drug Discov. *11*, 633–652.

Firszt, R., Francisco, D., Church, T.D., Thomas, J.M., Ingram, J.L., and Kraft, M. (2014). Interleukin-13 induces collagen type-1 expression through matrix metalloproteinase-2 and transforming growth factor-β1 in airway fibroblasts in asthma. Eur. Respir. J. *43*, 464–473.

Gabriel, D.A., Muga, K., and Boothroyd, E.M. (1992). The effect of fibrin structure on fibrinolysis. J. Biol. Chem. *267*, 24259–24263.

592 Godin, J.-P., Ross, A.B., Cléroux, M., Pouteau, E., Montoliu, I., Moser, M., and Kochhar, S.
593 (2013). Natural carbon isotope abundance of plasma metabolites and liver tissue differs
594 between diabetic and non-diabetic Zucker diabetic fatty rats. PloS One *8*, e74866.

595 Halcox, J.P.J., Donald, A.E., Ellins, E., Witte, D.R., Shipley, M.J., Brunner, E.J., Marmot,
596 M.G., and Deanfield, J.E. (2009). Endothelial function predicts progression of carotid intima-
597 media thickness. Circulation *119*, 1005–1012.

598 Hotary, K.B., Yana, I., Sabeh, F., Li, X.-Y., Holmbeck, K., Birkedal-Hansen, H., Allen, E.D.,
599 Hiraoka, N., and Weiss, S.J. (2002). Matrix metalloproteinases (MMPs) regulate fibrin-
600 invasive activity via MT1-MMP-dependent and -independent processes. J. Exp. Med. *195*,
601 295–308.

602 Konings, J., Govers-Riemslag, J.W.P., Philippou, H., Mutch, N.J., Borissoff, J.I., Allan, P.,
603 Mohan, S., Tans, G., Ten Cate, H., and Ariëns, R.A.S. (2011). Factor XIIa regulates the
604 structure of the fibrin clot independently of thrombin generation through direct interaction
605 with fibrin. Blood *118*, 3942–3951.

606 Konstantinides, S., Schäfer, K., Koschnick, S., and Loskutoff, D.J. (2001). Leptin-dependent
607 platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic
608 disease in obesity. J. Clin. Invest. *108*, 1533–1540.

609 Kotronen, A., Joutsi-Korhonen, L., Sevastianova, K., Bergholm, R., Hakkarainen, A.,
610 Pietiläinen, K.H., Lundbom, N., Rissanen, A., Lassila, R., and Yki-Järvinen, H. (2011).
611 Increased coagulation factor VIII, IX, XI and XII activities in non-alcoholic fatty liver
612 disease. Liver Int. Off. J. Int. Assoc. Study Liver *31*, 176–183.

613 Kumar, R., Béguin, S., and Hemker, H.C. (1994). The influence of fibrinogen and fibrin on
614 thrombin generation--evidence for feedback activation of the clotting system by clot bound
615 thrombin. Thromb. Haemost. *72*, 713–721.

616 Lacolley, P., Regnault, V., Nicoletti, A., Li, Z., and Michel, J.-B. (2012). The vascular smooth
617 muscle cell in arterial pathology: a cell that can take on multiple roles. Cardiovasc. Res. *95*,
618 194–204.

619 Lee, S., Park, Y., Dellsperger, K.C., and Zhang, C. (2011). Exercise training improves
620 endothelial function via adiponectin-dependent and independent pathways in type 2 diabetic
621 mice. Am. J. Physiol. Heart Circ. Physiol. *301*, H306–H314.

622 Li, Z., Froehlich, J., Galis, Z.S., and Lakatta, E.G. (1999). Increased expression of matrix
623 metalloproteinase-2 in the thickened intima of aged rats. Hypertens. Dallas Tex 1979 *33*, 116–
624 123.

625 Libby, P., Ordovas, J.M., Auger, K.R., Robbins, A.H., Birinyi, L.K., and Dinarello, C.A.
626 (1986). Endotoxin and tumor necrosis factor induce interleukin-1 gene expression in adult
627 human vascular endothelial cells. Am. J. Pathol. *124*, 179–185.

628 Lim, H.S., Lip, G.Y.H., and Blann, A.D. (2004). Plasma von Willebrand factor and the
629 development of the metabolic syndrome in patients with hypertension. J. Clin. Endocrinol.
630 Metab. *89*, 5377–5381.

631 Lominadze, D., Dean, W.L., Tyagi, S.C., and Roberts, A.M. (2010). Mechanisms of
632 fibrinogen-induced microvascular dysfunction during cardiovascular disease. Acta Physiol.
633 Oxf. Engl. *198*, 1–13.

634 Lu, P., Liu, J., Liu, N., Guo, F., Ji, Y., and Pang, X. (2011). Pro-inflammatory effect of
635 fibrinogen and FDP on vascular smooth muscle cells by IL-6, TNF-α and iNOS. Life Sci. *88*,
636 839–845.

637 Mao, X., Said, R., Louis, H., Max, J.-P., Bourhim, M., Challande, P., Wahl, D., Li, Z.,
638 Regnault, V., and Lacolley, P. (2012). Cyclic stretch-induced thrombin generation by rat
639 vascular smooth muscle cells is mediated by the integrin αvβ3 pathway. Cardiovasc. Res. *96*,
640 513–523.

641 Matsuzawa, Y., Funahashi, T., Kihara, S., and Shimomura, I. (2004). Adiponectin and
642 metabolic syndrome. Arterioscler. Thromb. Vasc. Biol. *24*, 29–33.

643 Membre, A., Wahl, D., Latger-Cannard, V., Max, J.-P., Lacolley, P., Lecompte, T., and
644 Regnault, V. (2008). The effect of platelet activation on the hypercoagulability induced by
645 murine monoclonal antiphospholipid antibodies. Haematologica *93*, 566–573.

646 Mook, O.R.F., Van Overbeek, C., Ackema, E.G., Van Maldegem, F., and Frederiks, W.M.
647 (2003). In situ localization of gelatinolytic activity in the extracellular matrix of metastases of
648 colon cancer in rat liver using quenched fluorogenic DQ-gelatin. J. Histochem. Cytochem.
649 Off. J. Histochem. Soc. *51*, 821–829.

650 Morozumi, T., Sharma, A., and De Nardin, E. (2009). The functional effects of the -455G/A
651 polymorphism on the IL-6-induced expression of the beta-fibrinogen gene may be due to
652 linkage disequilibrium with other functional polymorphisms. Immunol. Invest. *38*, 311–323.

653 Ninivaggi, M., Apitz-Castro, R., Dargaud, Y., de Laat, B., Hemker, H.C., and Lindhout, T.
654 (2012). Whole-blood thrombin generation monitored with a calibrated automated
655 thrombogram-based assay. Clin. Chem. *58*, 1252–1259.

656 Pacheco, Y.M., Bermúdez, B., López, S., Abia, R., Villar, J., and Muriana, F.J.G. (2006).
657 Ratio of oleic to palmitic acid is a dietary determinant of thrombogenic and fibrinolytic
658 factors during the postprandial state in men. Am. J. Clin. Nutr. *84*, 342–349.

659 Pandolfi, A., Cetrullo, D., Polishuck, R., Alberta, M.M., Calafiore, A., Pellegrini, G.,
660 Vitacolonna, E., Capani, F., and Consoli, A. (2001). Plasminogen activator inhibitor type 1 is
661 increased in the arterial wall of type II diabetic subjects. Arterioscler. Thromb. Vasc. Biol. *21*,
662 1378–1382.

663 Paul, W., Queen, L.R., Page, C.P., and Ferro, A. (2007). Increased platelet aggregation in
664 vivo in the Zucker Diabetic Fatty rat: differences from the streptozotocin diabetic rat. Br. J.
665 Pharmacol. *150*, 105–111.

666 Petrini, S., Neri, T., Lombardi, S., Cordazzo, C., Balìa, C., Scalise, V., Paggiaro, P.,
667 Pedrinelli, R., and Celi, A. (2016). Leptin induces the generation of procoagulant, tissue
668 factor bearing microparticles by human peripheral blood mononuclear cells. Biochim.
669 Biophys. Acta *1860*, 1354–1361.

670 Phillips, M.S., Liu, Q., Hammond, H.A., Dugan, V., Hey, P.J., Caskey, C.J., and Hess, J.F.
671 (1996). Leptin receptor missense mutation in the fatty Zucker rat. Nat. Genet. *13*, 18–19.

672 Regnault, V., Hemker, H.C., Wahl, D., and Lecompte, T. (2004). Phenotyping the
673 haemostatic system by thrombography--potential for the estimation of thrombotic risk.
674 Thromb. Res. *114*, 539–545.

675 Restituto, P., Colina, I., Varo, J.J., and Varo, N. (2010). Adiponectin diminishes platelet
676 aggregation and sCD40L release. Potential role in the metabolic syndrome. Am. J. Physiol.
677 Endocrinol. Metab. *298*, E1072–E1077.

678 Ridker, P.M., Everett, B.M., Thuren, T., MacFadyen, J.G., Chang, W.H., Ballantyne, C.,
679 Fonseca, F., Nicolau, J., Koenig, W., Anker, S.D., et al. (2017). Antiinflammatory Therapy
680 with Canakinumab for Atherosclerotic Disease. N. Engl. J. Med. *377*, 1119–1131.

681 Samad, F., Pandey, M., and Loskutoff, D.J. (2001). Regulation of tissue factor gene
682 expression in obesity. Blood *98*, 3353–3358.

683 Semeraro, F., Ammollo, C.T., Morrissey, J.H., Dale, G.L., Friese, P., Esmon, N.L., and
684 Esmon, C.T. (2011). Extracellular histones promote thrombin generation through platelet-
685 dependent mechanisms: involvement of platelet TLR2 and TLR4. Blood *118*, 1952–1961.

686 Shang, J., Chen, Z., Wang, M., Li, Q., Feng, W., Wu, Y., Wu, W., Graziano, M.P., and
687 Chintala, M. (2014). Zucker Diabetic Fatty rats exhibit hypercoagulability and accelerated
688 thrombus formation in the Arterio-Venous shunt model of thrombosis. Thromb. Res. *134*,
689 433–439.

690 Shrestha, C., Ito, T., Kawahara, K., Shrestha, B., Yamakuchi, M., Hashiguchi, T., and
691 Maruyama, I. (2013). Saturated fatty acid palmitate induces extracellular release of histone
692 H3: a possible mechanistic basis for high-fat diet-induced inflammation and thrombosis.
693 Biochem. Biophys. Res. Commun. *437*, 573–578.

694 Simon, J.A., Hodgkins, M.L., Browner, W.S., Neuhaus, J.M., Bernert, J.T., and Hulley, S.B.
695 (1995). Serum fatty acids and the risk of coronary heart disease. Am. J. Epidemiol. *142*, 469–
696 476.

697 Sloboda, N., Fève, B., Thornton, S.N., Nzietchueng, R., Regnault, V., Simon, G., Labat, C.,
698 Louis, H., Max, J.-P., Muscat, A., et al. (2012). Fatty acids impair endothelium-dependent
699 vasorelaxation: a link between obesity and arterial stiffness in very old Zucker rats. J.
700 Gerontol. A. Biol. Sci. Med. Sci. *67*, 927–938.

701 Sonnenberg, G.E., Krakower, G.R., and Kissebah, A.H. (2004). A novel pathway to the
702 manifestations of metabolic syndrome. Obes. Res. *12*, 180–186.

703 Suehiro, A., Wakabayashi, I., Uchida, K., Yamashita, T., and Yamamoto, J. (2012). Impaired
704 spontaneous thrombolytic activity measured by global thrombosis test in males with
705 metabolic syndrome. Thromb. Res. *129*, 499–501.

706 Wagenvoord, R.J., Hendrix, H.H., Kai, H., and Hemker, H.C. (1994). A chromogenic test to
707 determine the procoagulant phospholipids in platelet-rich plasma and whole blood. Thromb.
708 Haemost. *72*, 582–587.

709 Wakil, S.J., and Abu-Elheiga, L.A. (2009). Fatty acid metabolism: target for metabolic
710 syndrome. J. Lipid Res. *50*, S138–S143.

711 Wang, M., Zhang, J., Jiang, L.-Q., Spinetti, G., Pintus, G., Monticone, R., Kolodgie, F.D.,
712 Virmani, R., and Lakatta, E.G. (2007). Proinflammatory profile within the grossly normal
713 aged human aortic wall. Hypertens. Dallas Tex 1979 *50*, 219–227.

714 Wang, M.Y., Zhou, Y.T., Newgard, C.B., and Unger, R.H. (1996). A novel leptin receptor
715 isoform in rat. FEBS Lett. *392*, 87–90.

716 Wanninger, J., Walter, R., Bauer, S., Eisinger, K., Schäffler, A., Dorn, C., Weiss, T.S.,
717 Hellerbrand, C., and Buechler, C. (2011). MMP-9 activity is increased by adiponectin in
718 primary human hepatocytes but even negatively correlates with serum adiponectin in a rodent
719 model of non-alcoholic steatohepatitis. Exp. Mol. Pathol. *91*, 603–607.

720 Weiss, T.W., Arnesen, H., and Seljeflot, I. (2013). Components of the interleukin-6
721 transsignalling system are associated with the metabolic syndrome, endothelial dysfunction
722 and arterial stiffness. Metabolism. *62*, 1008–1013.

723 Wolberg, A.S. (2007). Thrombin generation and fibrin clot structure. Blood Rev. *21*, 131–
724 142.

725 Zhou, Y.-P., Madjidi, A., Wilson, M.E., Nothhelfer, D.A., Johnson, J.H., Palma, J.F.,
726 Schweitzer, A., Burant, C., Blume, J.E., and Johnson, J.D. (2005). Matrix metalloproteinases
727 contribute to insulin insufficiency in Zucker diabetic fatty rats. Diabetes *54*, 2612–2619.

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Figure legends

Figure 1: Platelet aggregation, thrombin generation and fibrinolysis in LZR and MSZR rats. (A) Mean maximum aggregation in washed platelets in response to collagen (5 µg/ml) and in (B) platelet-rich plasma (PRP) in response to ADP (5 µM), with the platelet count adjusted to 200×10^9 platelets/l. (C) Calibrated automated thrombinography (CAT) in rat plasma. Mean thrombin generation curves in platelet free plasma (PFP) triggered by 5 pM tissue factor in LZR and MSZR at 25 and 80 weeks of age. (D) Endogenous thrombin potential (ETP) in PFP and PRP of 25 and 80 week-old LZR and MSZR, expressed as ratios of values for 25 week-old LZR. (E) Ultrastructure of fibrin fibers was visualized by scanning electron microscopy. Pictures were made at 10.000x magnification. (F, G) Fiber thickness and fiber density of fibrin clot in LZR and MSZR. (H) ELISA results of PAI-1 measured in PFP (n=17-19). (I) Representative curves of fibrinolytic tests in PFP in LZR and MSZR. (J, K) Half-lysis time and maximal lysis speed of fibrinolytic tests in LZR and MSZR. Results are mean ± standard error of the mean (n=7-11). * $p<0.05$ *vs* LZR at the same age; # $p<0.05$ *vs* 25 week-old rats in the same strain.

Figure 2: Effect of fibrinogen, adipokines and free fatty acids on thrombin generation. (A) Correlation between ETP and plasma fibrinogen concentration of 25 and 80 week-old LZR and MSZR, ($r = 0.069$, $p = 0.01$). (B) ETP values in 25 week-old LZR platelet free plasma supplemented with 0.5, 1.0, 2.0 or 2.5 g/l fibrinogen. (C to F) ETP values, expressed as ratios of values in presence of adipokines or free fatty acids to those obtained with no addition for each group, in platelet free plasma supplemented with 0.05, 0.1 or 1.0 ng/ml leptin (C), with 2, 4, or 8 µg/ml adiponectin (D), with 0.75, 1.5 or 3 mg/ml of linoleic acid (E) or with 0.75 1.5 or 3 mg/ml of palmitic acid (F). Results are mean ± standard error of the mean (n=11-16). * $p<0.05$ vs no addition.

756 **Figure 3: Plasma cytokine array in Zucker rats.** (A) Cytokine arrays of pooled platelet free
757 plasma from 25 and 80 week-old MSZR and LZR. Relative chemoluminescence compared to
758 25 week-old LZR was measured. (B) Unchanged cytokines, (C) cytokines modified with age,
759 (D) with MetS, or (E) both with MetS and age. ELISAs results for IL-1β (F) and IL-13 (G)
760 measured in PFP (n=14-18), results are mean ± standard error of the mean, * $p<0.05$ *vs* LZR
761 at the same age; # $p<0.05$ *vs* 25 week-old rats in the same strain. VEGF, vascular endothelial
762 growth factor; CINC-1, cytokine-induced neutrophil chemoattractant 1; CINC-3, cytokine-
763 induced neutrophil chemoattractant 3; GM-CSF, granulocyte macrophage colony stimulating
764 factor; MIP, Macrophage Inflammatory Protein; MIG, C-X-C motif ligand 9; IP-10,
765 interferon gamma-induced protein 10; CNTF, ciliary neurotrophic factor; INFγ, interferon γ;
766 IL, interleukin.
767
768 **Figure 4: Role of smooth muscle cells in thrombin generation.** (A) ETP values measured at
769 the surface of vascular smooth muscle cells (VSMCs) from LZR and MSZR, with LZR or
770 MSZR platelet free plasma (PFP), and with or without 1.5 g/l exogenous added palmitic acid
771 (PAL). Results are mean ± standard error of the mean, n=3 with 6 wells per condition per
772 experiment. * $p<0.05$ vs LZR VSMC, # $p<0.05$ vs LRZ PFP and LRZ VMSC. (B) VSMC
773 associated procoagulant activity reported as phosphatidylserine (PS) equivalent in LRZ and
774 MSZR. Results are mean ± standard error of the mean (n=25). * $p<0.05$ *vs* LZR. (C) Typical
775 Western blot and (D) quantification analysis of VSMC differentiation markers (αSMA, SM-
776 MHC and smoothelin) and integrin subunits (α_v and β_3) in cultured VSMCs. Results,
777 expressed as fold change vs VSMCs from LZR, are mean ± standard error of the mean (n=6).
778 * $p<0.05$, MSZR *vs* LZR. (E) Representative images of gelatinolytic metalloproteinase
779 activity in the aorta was measured using *in situ* gelatin zymography for each group of Zucker
780 rats. Fluorescence as marker for intra-plaque gelatinolytic activity was quantified. Nuclei
781 were visualized by DAPI staining. (F) Average wall fluorescence of the gelatinolytic

782 metalloproteinase activity in the aorta. (G) Representative images of zymography gels of LZR
783 and MSZR VSCMCs supernatant at 4h, 8h and 20h. (H) Relative MMP-2 activity in LZR and
784 MSZR VSMC supernatant at 4h, 8h and 20h. Results are mean ± standard error of the mean
785 (*n*=5). * $p<0.05$, MSZR *vs* LZR. ELISAs results of MMP-9 (I) and VCAM-1 (J) measured in
786 PFP (n= 17-22). * $p<0.05$ *vs* LZR at the same age; # $p<0.05$ *vs* 25 week-old rats in the same
787 strain.
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789 **Table 1**: **Blood coagulation parameters and thrombin generation parameters of LZR**

790 **and MSZR at 25 and 80 weeks of age.**

	25 week-old		**80 week-old**		**ANOVA**		
	LZR	**MSZR**	**LZR**	**MSZR**	**Strain**	**Age**	**Interaction**
n	9	10	12	9			
Platelets (10^3/mm^3)	574±37	789±34*	633±29	834±63*	≤ 0.0001	0.009	0.013
F1+2 (pmol/l)	4.1 ± 0.5	7.9 ± 1.0 *	5.8 ± 1.2	5.5 ± 0.9	0.009	0.7	0.05
TF (pM)	0.3 ± 0.1	12.2 ± 1.7 *	2.0 ± 0.4	9.9 ± 1.5 *	≤ 0.0001	0.8	0.09
TFPI activity (U/ml)	4.9 ± 0.2	11.2 ± 0.2*	5.4 ± 0.2	9.9 ± 0.6* #	≤ 0.0001	0.3	0.01
FVIII (%)	104 28	190 34	124 28	466 52* #	≤ 0.0001	0.002	0.001
Prothrombin (%)	94 ± 3	223 ± 19 *	155 ± 14 #	264 ± 16 *	≤ 0.0001	0.002	0.5
AT (%)	129 ± 2	125 ± 2	127 ± 1	123 ± 3	0.04	0.5	0.9
Fibrinogen (g/l)	2.8 ± 0.1	4.0 ± 0.2 *	3.1 ± 0.1	4.9 ± 0.2 * #	≤ 0.0001	0.0003	0.2
n	11	11	10	7			
Lag time (min)	1.5 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	0.09	0.4	0.6
Peak (nM)	99 ± 8	121 ± 9	102 ± 10	117 ± 15	0.07	0.98	0.7
Time to peak (min)	4.4 ± 0.1	5.2 ± 0.3 *	4.1 ± 0.1	5.3 ± 0.2 *	≤ 0.0001	0.6	0.3
ETP (nM.min)	395 ± 37	549 ± 52 *	362 ± 34	553 ± 76 *	0.001	0.8	0.8
Velocity (nM/min)	35 ± 3	37 ± 4	40 ± 4	31 ± 4	0.6	0.98	0.2

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793 Results are mean ± standard error to the mean. * p < 0.05, SMZR *vs* LZR at the same age; # p

794 < 0.05, 80 *vs* 25 week-old rats in the same strain. F1+2, fragment 1+2; TF, tissue factor; TFPI,

795 tissue factor pathway inhibitor; AT, antithrombin. ETP, endogenous thrombin potential.