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Biocompatible modified water as a non-pharmaceutical approach to prevent metabolic syndrome features in obesogenic diet-fed mice

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ABSTRACT

The prevalence of metabolic syndrome (MetS), elevating cardiovascular risks, is increasing worldwide, with no available global therapeutic options. The intake of plain, mineral or biocompatible modified waters was shown to prevent some MetS features. This study was designed to analyze, in mice fed a high fat and sucrose diet (HFSD), the effects on MetS features of the daily intake of a reverse osmosed, weakly remineralized, water (OW) and of an OW dynamized by a physical processing (ODW), compared to tap water (TW). The HFSD was effective at inducing major features of MetS such as obesity, hepatic steatosis and inflammation, blood dyslipidemia, systemic glucose intolerance and muscle insulin resistance. Compared to TW, OW intake decreased hepatic fibrosis and inflammation, and mitigated hepatic steatosis and dyslipidemia. ODW intake further improved skeletal muscle insulin sensitivity and systemic glucose tolerance. This study highlights the deleterious metabolic impacts of the daily intake of TW, in combination with a high energy diet, and its possible involvement in MetS prevalence increase. In addition, it demonstrates that biocompatible modified water may be promising non-pharmaceutical, cost-effective tools for nutritional approaches in the treatment of MetS.

1. Introduction

The metabolic syndrome (MetS) is a cluster of metabolic disorders that include central obesity, high blood pressure, triglycerides (TG) and fasting glycaemia, low HDL cholesterol and insulin resistance (Alberti et al., 2009). Non-alcoholic fatty liver disease (NAFLD) is a frequent hepatic manifestation co-existing with MetS and is characterized by fatty infiltration of the liver in the absence of alcohol use or other known liver diseases. It may evolve to more severe forms such as non-alcoholic steatohepatitis (NASH), liver cirrhosis or hepatocellular carcinoma (Marchesini et al., 2003). Each of these metabolic disturbances is considered as a risk factor for cardiovascular diseases and increased mortality (O'Neill and O'Driscoll, 2015). The increasing prevalence of MetS over the past decades generates an urgent need for novel strategies to prevent or treat this global epidemic. MetS treatment may involve targeted (often multiple) pharmacological therapy toward each risk factor. However, the main recommendations for the greatest benefits in MetS prevention and management consist in non-pharmacological approaches with lifestyle changes such as overall healthier diet and daily physical activity practice (American Heart Association Nutrition Committee et al., 2006; Pérez-Martínez et al., 2017). Indeed, non-pharmaceutical nutritional approaches primarily target caloric restriction, in association with the chronic consumption of nutraceuticals such as phytochemicals (soluble fibers from psyllium, curcumin from curcuma, alliin from garlic, cinnamon phytochemicals, berberine, vegetable omega-3 polyunsaturated fatty acids, catechins and flavonols from green tea and cocoa, resveratrol), and were shown to successfully prevent or treat one or more features of the MetS and NAFLD (Cicero et al., 2018; Cicero and Colletti, 2016; Minich and Bland, 2008; Rochlani et al., 2017).

Water is a vital nutrient and the main constituent of all living beings. At the whole-body level, water acts as a multifunctional component (Popkin et al., 2010). In cells, water molecules are confined and submitted to structural effects, generating an interfacial or biological water known to be an active biomolecule involved in the structure, dynamic, interaction and function of biological macromolecules (Ball, 2017; Chaplin, 2006). Despite its major place in cell biology, water is often ignored in dietary recommendations and there are discrepancies

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Abbreviations

Akt	protein kinase B
P-Akt	phosphorylated Akt
AUC	area under curve
ATWATER	metabolisable energy
AVP	arginine vasopressin
αSMA	alpha smooth muscle actin
BW	body weight
CCL2	chemokine ligand 2
CD	chow diet
CD36	cluster of differentiation 36
CD68	cluster of differentiation 68
COL1A1	collagen type I alpha 1
COL3A1	collagen type III alpha 1
DNA	deoxyribonucleic acid
ERW	electrochemically reduced water
EZ	exclusion zone water
Σ	energy
Fasn	fatty acid synthase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HFSD	high fat and sucrose diet
HOMA-IR	homeostasic model assessment of insulin resistance
IL1RA	interleukin-1 receptor antagonist
IL1β	interleukin-1 beta
IL6	interleukin-6
IL10	interleukin-10
IPGTT	intraperitoneal glucose test tolerance
MetS	metabolic syndrome
MMP2	matrix metalloproteinase-2
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acids
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD score
NASH	non-alcoholic steatohepatitis
NFE	nitrogen-free extract
OW	osmosed water
ODW	osmosed dynamized water
PBS	phosphate buffer saline
PLIN2	perilipin 2
PPARγ	peroxisome proliferator-activated receptor gamma
PUFA	polyunsaturated fatty acids
RER	respiratory exchange ratio–
ROS	reactive oxygen species
Saa1	serum amyloid A1
SFA	saturated fatty acids
TGFβ	transforming growth factor beta
TIMP1	metallopeptidase inhibitor 1
TNFα	tumor necrosis factor alpha
TG	triglycerides
T2DM	type 2 diabetes mellitus
TW	tap water
UFA	unsaturated fatty acids
WAT	white adipose tissues

regarding the amount, the type and the quality of our drinking water intake for optimum health.

Arginine vasopressin (AVP) is an antidiuretic hormone regulating fluid balance through water reabsorption in the kidney (Park and Kwon, 2015) and water absorption in the intestine (Pais et al., 2016), by controlling water permeability through the regulation of water channels named aquaporins. Recent evidences show that the amount of water intake and thus, the vasopressin-hydration axis, regulates glucose homeostasis and hepatic steatosis. Indeed, AVP has been shown to stimulate hepatic gluconeogenesis and glycogenolysis as well as insulin and glucagon secretion from pancreatic islets (Abu-Basha et al., 2002; Keppens and de Wulf, 1979). In addition, it is demonstrated that low water intake increases the risk of developing hyperglycemia (Johnson et al., 2017; Roussel et al., 2011). Accordingly, high water intake supplementation has been correlated with significant decreases in plasma AVP and its reliable marker copeptin (Lemetais et al., 2018), to a decrease in fasting plasma glucose and type 2 diabetes mellitus (T2DM) risks in patients (Carroll et al., 2015; Enhörning et al., 2018), and an improvement in hepatic steatosis and lipid metabolism in obese rats (Taveau et al., 2015). Water composition has also been shown to decrease cardiometabolic risk factors such as fasting glucose (Naumann et al., 2017) and dyslipidemia with naturally high-bicarbonated mineral water in hypercholesterolemic (Toxqui and Vaquero, 2016) and postmenopausal patients (Schoppen et al., 2004).

In addition to water intake and composition, the health-beneficial properties of biocompatible water modified by physical processing have also been studied. Biocompatible electrochemically reduced water (ERW), generated by electrolysis, are characterized by high level of activated dihydrogen molecules (H_2) efficiently scavenging reactive oxygen species (ROS) (Ohsawa et al., 2007). These ERW have been shown to improve oxidative stress-related diseases. In fact, accumulated evidences have demonstrated that ERW prevent obesity and diabetes in various rodent models (Kamimura et al., 2011), as well as oxidative stress associated with MetS (Nakao et al., 2010) and insulin resistance in T2DM patients (Kajiyama et al., 2008). Finally, biocompatible magnetized water, generated by magnetic field application, prevented diabetes features in streptozotocin-treated rats (Lee and Kang, 2013).

From these observations, the purpose of this study was to test the ability of a biocompatible reverse osmosed water (OW) and a new biocompatible dynamized water (ODW), compared with tap water (TW), to prevent cardiometabolic risk factors associated to MetS in obesogenic high fat and sucrose diet (HFSD)-fed mice.

2. Materials and methods

2.1. Animal ethics

All procedures conformed to European Parliament Directive 2010/63/EU, the 22 September 2010 Council on animal protection, and NIH Guidelines for the Care and Use of Laboratory Animals. The project was approved by the committee for Animal Care of Montpellier-Languedoc-Roussillon (N° CEEA-LR-12159).

2.2. Biocompatible modified water production and water properties

Osmosed and osmosed dynamized water generating devices were designed and installed by Natarys (Plessé, France). TW was obtained from the same laboratory tap. OW was obtained after TW reverse osmosis filtration, using a system composed of: 1) a 5 μm diameter sediment filter (Ceasa A-255500); 2) an activated carbon filter (Ceasa A-255700); 3) a nanometer diameter polyamide membrane (Ceasa RO 75 GDP); 4) a post final filtration, through a cartridge made of activated carbon and fossilized algae (Ceasa A-251050) to obtain necessary pH and Ca^{2+} and Mg^{2+} mineralization for the generation of biocompatible drinking water and to avoid potential adverse health effects (World Health Organization, 2009). Since reverse osmosis also affects the stereo-chemical structure of water molecules, OW received an electro-vibratory treatment called dynamization (based on Marcel Violet's patent N° 1.142.722) which generated an ODW. Briefly, the dynamization device captures high frequency harmonic waves from the main electrical network, filters them through a capacitor which transfers them to the water through a silver electrode. The pH, conductivity (μS/cm),

redox (Rel. mV) and dissolved oxygen (%) of water were measured weekly and waters ionic composition (mg/L) were determined by colorimetric assays and inductively coupled plasma associated to optical emission spectrometry (ICP-OES) (CIRAD, Montpellier, France) (Table 1).

2.3. Experimental design

Four weeks old male C57BL/6J mice (Janvier, Le Genest-Saint-Isle, France) were housed in the laboratory's animal facility, with a pathogen free and controlled environment (21 ± 1 °C; humidity 60%; 12-h light cycle). After one week of acclimatization (day 0), animals were randomly assigned to four groups (n = 10 each) as followed: 1) a control group fed with a standard chow diet (CD) (A04; Safe, Augy, France) and TW (CD + TW); 2) a MetS-induced group fed with a high-fat and sucrose diet (HFSD) (260HF diet (U8978 version 19); Safe, Augy, France) and TW, (HFSD + TW); 3) a MetS-induced group fed with a HFSD and OW, (HFSD + OW); and 4) a MetS-induced group fed with a HFSD and ODW, (HFSD + ODW). Chow diet (A04) and HFSD (260HF) manufacturer's compositions are presented in Table 2. Food and water were given *ad libitum* for 12 weeks. Water was changed every day and food every two days. Food and water intakes were measured, corrected for drying, and used to calculate daily caloric intake (Kcal). Body weight (BW) was measured weekly and final BW was obtained prior to sacrifice after 12 weeks of feeding on 12h-fasted animals. Final BW gain was calculated by subtracting final BW to BW at day 0.

2.4. Glucose tolerance test

One week prior to sacrifice, an intraperitoneal glucose tolerance test (IPGTT) was performed on 12h-fasted animals as previously described (Lambert et al., 2018). Briefly, after fasting, a glucose solution [2 g kg^{-1} BW saline (0.9% NaCl)] was administered intraperitoneally. Blood was drawn by tail snipping of the caudal vein. Blood glucose was measured using a commercial glucometer (Abbott, Papillon vision) before, and at 20, 40, 60, 90, and 120 min, after glucose injection. Area under curve (AUC) values were obtained using GraphPad PRISM software (version 6.0.7).

2.5. Mice sacrifice and biochemical analyses

One week after IPGTT, 12h-fasted mice were weighted and sacrificed by cervical dislocation. Blood samples were collected from the abdominal artery with a heparinized syringe and distributed into heparinized dry tubes. Tubes were centrifuged at 1000 g for 10 min at 4 °C, and serum samples were collected and stored at −80 °C prior to analyses. In parallel, the heart, liver, soleus and anterior tibialis muscles, and epididymal, perirenal, mesenteric and inguinal white adipose tissues were collected, rinsed and weighed. Serum or liver total cholesterol, LDL- and HDL-cholesterol (E2HL-100), triglycerides (ETGA-200), lactate (EFLLC-100), and glycogen (E2GN-100) levels were quantified by colorimetric determination kits (Bioassay Systems, CA, USA). Resistin (ab205574), insulin (ab100578), adiponectin (ab108785) and leptin (ab100718) levels were quantified using ELISA kits (Abcam, MA, USA).

2.6. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from liver (50 mg) samples using the Nucleospin RNA Kit (Macherey-Nagel, Germany) and cDNA was generated as previously described (Lambert et al., 2018). qPCR mix was composed as followed: 2 µL of 1:10-diluted cDNA (10 ng), 0.5 µL of each primer (0.5 µM final concentration), 5 µL 2X concentrated LightCycler1 480 SYBR Green I Master and 2 µL PCR Grade water for a final volume of 10 µL. Specific primers (Eurofins Scientific, Ebersberg, Germany) for sequences of interest are listed in Supplemental Table S1 mRNA expression was quantified according to the comparative cycle threshold method, using Ct values in formula 2[Ct target gene—Ct reference gene] (2ΔCt). Data are expressed normalized to the CD + TW group.

2.7. Western blot analyses

Immunoblotting was performed on soleus muscle samples (≈15 mg) incubated in phosphate buffer saline (PBS) or in PBS with 1 µM insulin (15 min; 37 °C). Samples were homogenized using a manual polytron instrument and an ice-cold lysis buffer containing phosphatase and protease inhibitors (Sigma-Aldrich). Solubilized proteins were separated using SDS-PAGE electrophoresis and revealed overnight using the primary antibodies for total protein kinase B (Akt) (Akt; ab6672) and phosphorylated-Akt (P-Akt, Ser473; ab66579) from Abcam. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (s-c81178; Santa Cruz Biotechnology) was used for loading control. Bands were revealed and quantified with the Odyssey system (LI-COR Biosciences, Lincoln, Nebraska).

2.8. Histology

For histology, two duplicated paraffin liver sections, spaced 5 mm apart, were obtained from the left lateral lobe of each animal. Sections (5 µm) were stained with hematoxylin-eosin or picro-sirius red by standard procedures and were examined using bright field microscopy. Hematoxylin, eosin, picro-sirius red stained, and unstained areas were quantified, using GNU Image Manipulation Software (GIMP2.8), from three different fields (200X magnification; without tissue lesion/blood vessel lumen) for each section, generating 6 duplicated measurements for each animal. A mean value of these measurements was performed to obtain a representative value for each animal. Lipid droplets distribution, based on droplets surface area (small droplets < 10 μm^2 vs. large droplets > 10 μm^2) was performed using ImageJ software.

2.9. Statistical analyses

The protocol (10 mice/group) was repeated three times over a year

Table 1
General properties and ionic composition of waters.

	TW	OW	ODW
General properties			
pH	7.18 ± 0.04	7.54 ± 0.08[a]	7.49 ± 0.08[a]
Conductivity (µS/cm)	734 ± 6	143 ± 6[a]	138 ± 6[a]
Redox (relative mV)	326 ± 28	305 ± 27	307 ± 28
Dissolved oxygen (%)	95.6 ± 0.3	94.4 ± 1.4	94.6 ± 1.6
Ionic composition (mg/l)			
Ca	130	13.1	10.5
Mg	9.1	6.6	6.4
K	1.72	< 0.04	< 0.04
Na	20	3.7	3.7
Fe	< LD	< LD	< LD
Mn	< LD	< LD	< LD
S	9.04	0.24	0.23
P	< LD	< LD	< LD
B	< LD	< LD	< LD
$N\text{-}NO_3$	0.85	0.27	0.28
$N\text{-}NH_4$	< LD	< LD	< LD
Cl	37.85	3.93	3.98
HCO_3	376.37	74.42	65.27
Total	585	102	90

Ca. total calcium. *Mg.* total magnesium. *K.* total potassium. *Na.* total sodium. *Fe.* total iron. *Mn.* total manganese. *S.* total sulfur. *P.* total phosphorus. *B.* total boron. *$N\text{-}NO_3$.* total nitric nitrogen. *$N\text{-}NH_4$.* total ammonia nitrogen. *Cl.* total chorine. *HCO_3.* total bicarbonate. < LD: below detection level. Data are mean ± S.E.M. (n = 20). a, vs TW; p < 0.05.

Table 2
Control (A04) and high-fat and sucrose (260HF) diet compositions.

	A04	260HF
Starch (%)	43.5	14.5
Sugars (%)	3.2	20.2
Sucrose (%)	1.3	17.9
Protein (%)	16.1	19.9
Fat. (%)	3.1	35.9
Minerals (%)	4.6	4.2
Cellulose (%)	3.9	0.0
NFE (%)	60.4	37.2
Cholesterol (mg/kg)	0.0	869.7
ATWATER (Kcal/kg)	3417.0	5516.6
ATWATER (MJ/kg)	14.3	23.1
Σ from starch (%)	50.9	10.5
Σ from sugars (%)	3.7	14.7
Σ from protein (%)	18.8	14.4
Σ from fat (%)	8.2	58.6
Σ from NFE (%)	70.7	27.0
SFA (mg/kg)	6896.7	233 125.5
UFA (mg/kg)	21 909.5	93 825.3
MUFA (mg/kg)	5193.6	71 148.2
PUFA (mg/kg)	16 715.9	22 677.1
Total Omega-3 (mg/kg)	1628.8	3255.7
Total Omega-6 (mg/kg)	15 073.5	17 343.7

NFE. Nitrogen-free extract. *ATWATER*. Metabolisable energy. *Σ*. Energy. *SFA*. Saturated fatty acids. *UFA*. Unsaturated fatty acids. *MUFA*. Monounsaturated fatty acids. *PUFA*. Polyunsaturated fatty acids.

to ensure overtime reproducibility. Data from animals from the three different protocols were pooled. Devices generating OW or ODW were randomly labelled A and B for the experimenters and locked in a closet. Experiments were performed in a double-blinded randomized manner and water correspondence was revealed after final results delivery. Data are expressed as means ± standard errors of the mean (S.E.M). Statistics were performed using two-way (diet and water parameters) analysis of variance followed by Bonferroni post-test for two-group comparison, or Kruskal-Wallis one-way analysis of variance followed by Dunn's post hoc analyses for multiple-groups comparison. Data were analyzed using GraphPad PRISM software (USA). The limit of statistical significance was set at $p < 0.05$.

3. Results

3.1. ODW intake reduces basal metabolism

Both water and food intakes were reduced in all HFSD-fed groups compared to CD + TW (Fig. 1A). All animals receiving HFSD presented an increase in caloric intake and weight gain (Fig. 1A and B) with increased inguinal, epididymal, mesenteric and perirenal white adipose depots, compared to CD + TW (Table 3). Despite similar weight gain among all HFSD-fed groups, HFSD + OW presented a significant increase in water, food and caloric intakes. The basal metabolism was specifically decreased in HFSD + ODW compared to all other groups (Fig. 1C). The respiratory exchange ratio (RER) was significantly reduced in all HFSD-fed groups, compared to CD + TW, illustrating a greater reliance on lipid oxidation to provide energy (Fig. 1D).

3.2. OW and ODW intakes reduce serum dyslipidemia

Serum TG content was increased in all HFSD-fed groups compared to CD + TW (Table 4). However, it was significantly decreased in HFSD + OW and HFSD + ODW compared to HFSD + TW. No significant difference in total and LDL cholesterol was observed between groups. However, HDL cholesterol was significantly decreased in HFSD + TW compared to all others. Consequently, both calculated LDL/HDL and total cholesterol/HDL *ratios* were significantly increased in HFSD + TW. Adiponectin levels were increased in HFSD + OW compared to all other groups. Leptin levels were increased in all HFSD-fed groups compared to CD + TW. No difference was observed in resistin levels between all groups.

3.3. OW and ODW intakes reduce hepatic steatosis

Since MetS is often associated with NAFLD, liver alterations were investigated. Gross morphological differences were visible between whole livers from all HFSD-fed mice compared to CD + TW, but also between livers from HFSD + OW and HFSD + ODW mice compared to HFSD + TW. Similar observations were performed using hematoxylin-eosin staining on whole liver sections (Supplemental Figure S2). A more detail study of these sections revealed that livers from HFSD-fed mice were characterized by steatosis with marked accumulation of lipid droplets associated to hepatocellular ballooning degeneration, compared to CD + TW, (Fig. 2A, Supplemental Figure S2). In addition, fat

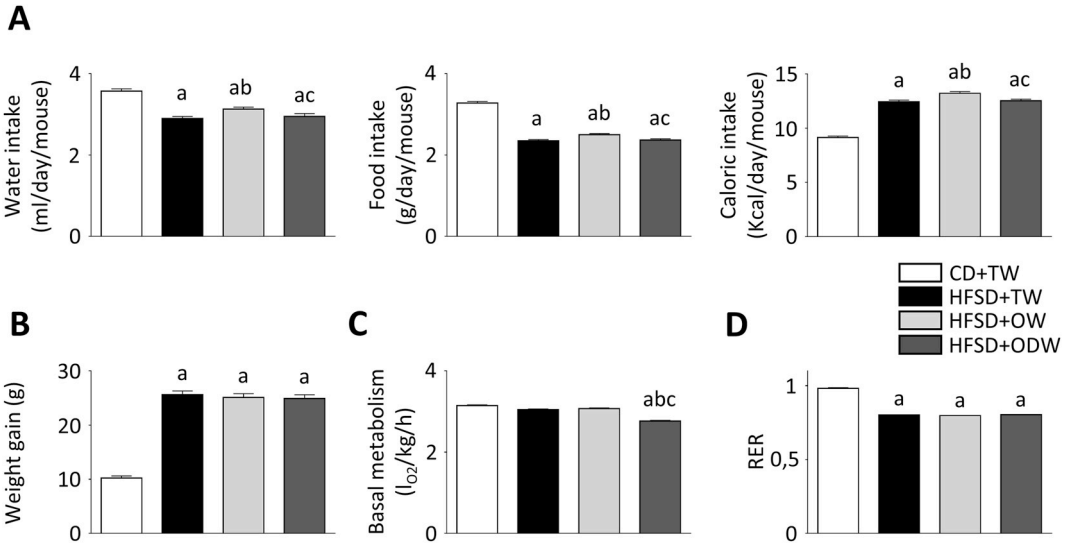

Fig. 1. Effects of modified water on intakes, weight gain and basal metabolism. A) *Left*: Water intake (ml/day/mouse), *Middle*: Food intake (g/day/mouse), *Right*: Caloric intake (Kcal/day/mouse), n = 24/group. B) Weight gain after 12 weeks (g), n = 30/group. C) Basal metabolism (l_{O2}/kg/h), n = 9/group. D) Respiratory exchange ratio, n = 9/group. Data are mean ± S.E.M. a, vs CD + TW; b, vs HFSD + TW; c, vs HFSD + OW; $p < 0.05$.

Table 3
Animal and organ weights.

	CD + TW	HFSD + TW	HFSD + OW	HFSD + ODW
Body weight (g)	29.89 ± 0.45	45.24 ± 0.68[a]	44.97 ± 0.64[a]	44.43 ± 0.73[a]
Liver weight (g)	1.24 ± 0.03	2.51 ± 0.13[a]	2.38 ± 0.13[a]	2.10 ± 0.11 [ab]
LW/BW (X100)	4.2 ± 1.1	5.5 ± 2.1[a]	5.1 ± 2.3[a]	4.8 ± 2.1[b]
Heart (mg)	160.9 ± 3.0	172.5 ± 4.1	186.2 ± 4.4[a]	179.7 ± 4.6[a]
Soleus muscle (mg)	22.81 ± 0.59	24.68 ± 0.64	27.70 ± 0.93[a]	25.91 ± 0.74[a]
Ant. Tib. muscle (mg)	125.0 ± 7.7	128.1 ± 4.2	140.7 ± 7.3	136.7 ± 10.2
Right kidney (mg)	171.0 ± 4.0	172.0 ± 4.0	186.0 ± 5.0	178.0 ± 6.0
Total WAT (g)	3.17 ± 0.08	8.26 ± 0.12[a]	8.21 ± 0.17[a]	8.21 ± 0.15[a]

LW/BW. Liver Weight/Body Weight. *Ant. Tib. muscle*. Anterior tibialis muscle. *Total WAT*. Total white adipose tissue. Data are mean ± S.E.M. n = 20–30/group. a, vs CD + TW; b, vs HFSD + TW; $p < 0.05$.

Table 4
Metabolic characteristics.

	CD + TW	HFSD + TW	HFSD + OW	HFSD + ODW
Glycaemia (mg/dl)	64.2 ± 2.4	120.3 ± 4.5[a]	111.8 ± 4.6[a]	111.0 ± 4.9[a]
Insulinemia (ng/ml)	0.52 ± 0.07	3.35 ± 0.36[a]	4.09 ± 0.40[a]	3.31 ± 0.56[a]
TG (mmol/l)	0.34 ± 0.03	1.61 ± 0.12[a]	1.05 ± 0.08 [ab]	1.19 ± 0.13 [ab]
Tot. chol. (mg/dl)	9.10 ± 0.62	8.32 ± 0.49	8.35 ± 0.65	8.69 ± 0.61
LDL chol. (mg/dl)	3.80 ± 0.25	3.77 ± 0.34	3.58 ± 0.9	3.21 ± 0.30
HDL chol. (mg/dl)	3.52 ± 0.39	2.49 ± 0.30[a]	3.45 ± 0.31[b]	3.39 ± 0.28[b]
LDL/HDL chol. index	1.18 ± 0.14	2.08 ± 0.49[a]	1.25 ± 0.25[b]	1.19 ± 0.12[b]
Tot. chol./HDL chol.	2.79 ± 0.23	5.19 ± 1.17[a]	3.21 ± 0.41[b]	3.17 ± 0.41[b]
Adiponectin (ng/ml)	128.9 ± 1.2	128.2 ± 0.9	135.1 ± 1.3 [ab]	130.4 ± 0.9[c]
Leptin (ng/ml)	0.58 ± 0.03	1.95 ± 0.02[a]	1.93 ± 0.02[a]	1.89 ± 0.03[a]
Resistin (μg/ml)	5.14 ± 1.18	5.13 ± 1.3	5.10 ± 1.1	5.21 ± 1.18
Lactate (μM)	279.2 ± 5.6	267.2 ± 6.6	292.6 ± 3.1[b]	285.4 ± 5.4[b]

TG. Triglycerides. *Tot. chol.* Total cholesterol. *LDL chol.* Low density lipoprotein cholesterol. *HDL chol.* High density lipoprotein cholesterol. All measurements were performed on 12h-fasted mice. Data are mean ± S.E.M. n = 20–30/group. a, vs CD + TW; b, vs HFSD + TW; $p < 0.05$.

area quantification showed an increase in livers from all HFSD-fed mice compared to CD + TW, but a significant decrease in livers from HFSD + OW and HFSD + ODW mice compared to HFSD + TW (Fig. 2B). In parallel, lipid droplets distribution demonstrated an increase in small and large droplets in HFSD-fed groups compared to CD + TW. Interestingly, both small and large droplets distributions

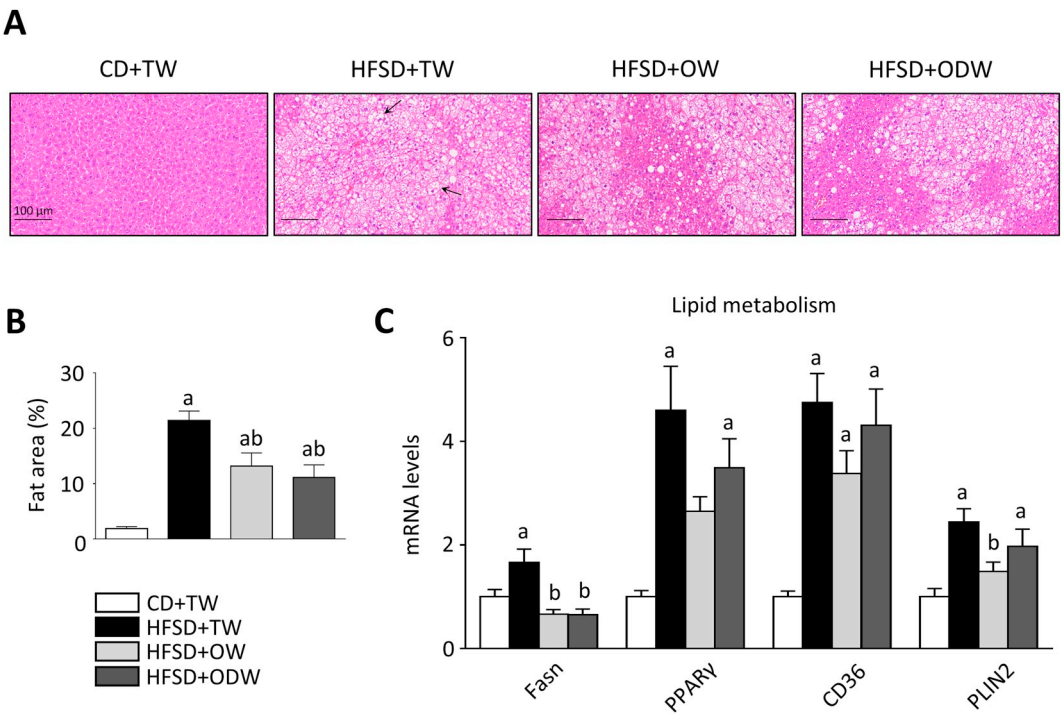

Fig. 2. Effects of modified water on hepatic steatosis. A) Representative hematoxylin-eosin staining of liver sections from each group, black arrows indicate hepatocellular ballooning (bar = 100 μm). B) Quantification of fat surface area (%), n = 10/group. C) mRNA expression level of markers of lipid metabolism: fatty acid synthase (Fasn), peroxisome proliferator-activated receptor gamma (PPARγ), cluster of differentiation 36 (CD36) and perilipin 2 (PLIN2), n = 9/group. Data are mean ± S.E.M. a, vs CD + TW; b, vs HFSD + TW; $p < 0.05$.

were uniformly decreased in HFSD + OW and HFSD + ODW, compared to HFSD + TW (Supplemental Table S3). Finally, a system of histological evaluation that encompasses the spectrum of NAFLD was used according to Kleiner et al. (2005). The NAFLD activity score (NAS) revealed 10 mice with a score < 3 (no NASH) in CD + TW, compared to 1 mouse with a score 3–4 (borderline) and 9 mice with a score ≥ 5 (NASH) in HFSD + TW. In both the HFSD + OW and HFSD + ODW groups, 5 mice were scored ≥5, 2 mice were scored 3–4 and 3 mice were scored < 3 (Supplemental Table S4), illustrating a decrease in NAS in HFSD + OW and HFSD + ODW, compared to HFSD + TW.

3.4. OW intake reduces molecular expression of lipogenesis markers

The mRNA expression of lipid metabolism markers was increased in HFSD + TW compared to CD + TW (Fig. 2C). HFSD + OW displayed reduced expressions of lipogenic factors fatty acid synthase (Fasn) and peroxisome proliferator-activated receptor gamma (PPARγ), and perilipin 2 (PLIN2) a member of lipid droplet protein family, compared to HFDS + TW. PPARγ and PLIN2 mRNA expression were also decreased in HFSD + ODW compared to HFSD + TW, but not in a significant fashion. Interestingly, HFSD + OW and CD + TW had similar expression levels of Fasn, PPARγ and PLIN2. The mRNA expression of CD36, involved in lipid uptake was significantly increased in all HFSD-fed groups compared to CD + TW, with no effect of OW or ODW intake.

3.5. OW and ODW intakes reduce liver fibrosis and molecular expression of fibrosis and inflammation markers

Obesity with visceral adiposity and insulin resistance is often associated with chronic low-grade systemic inflammation and accumulation of excess TG can increase the vulnerability of the liver to trigger hepatic fibrogenesis and inflammation. Thus, we investigated and quantified liver alterations using picro-sirius red staining of liver sections (Fig. 3A). Positive picro-sirius red area were mainly perivascular in our model and quantification demonstrated a significant decrease in HFSD + OW and HFSD + ODW compared to HFSD + TW (Fig. 3B), and no difference with CD + TW. The mRNA expression levels of fibrogenesis markers, except metallopeptidase inhibitor 1 (TIMP1), were not significantly different between CD + TW, HFSD + OW and HFSD + ODW. Moreover, ODW specifically decreased mRNA expression level of transforming growth factor beta (TGFβ) compared to HFSD + TW, whereas only a tendency was observed in OW (Fig. 3C).

Little lobular inflammation was observed while analyzing hematoxylin-eosin stained liver sections used for NAS. However, the mRNA

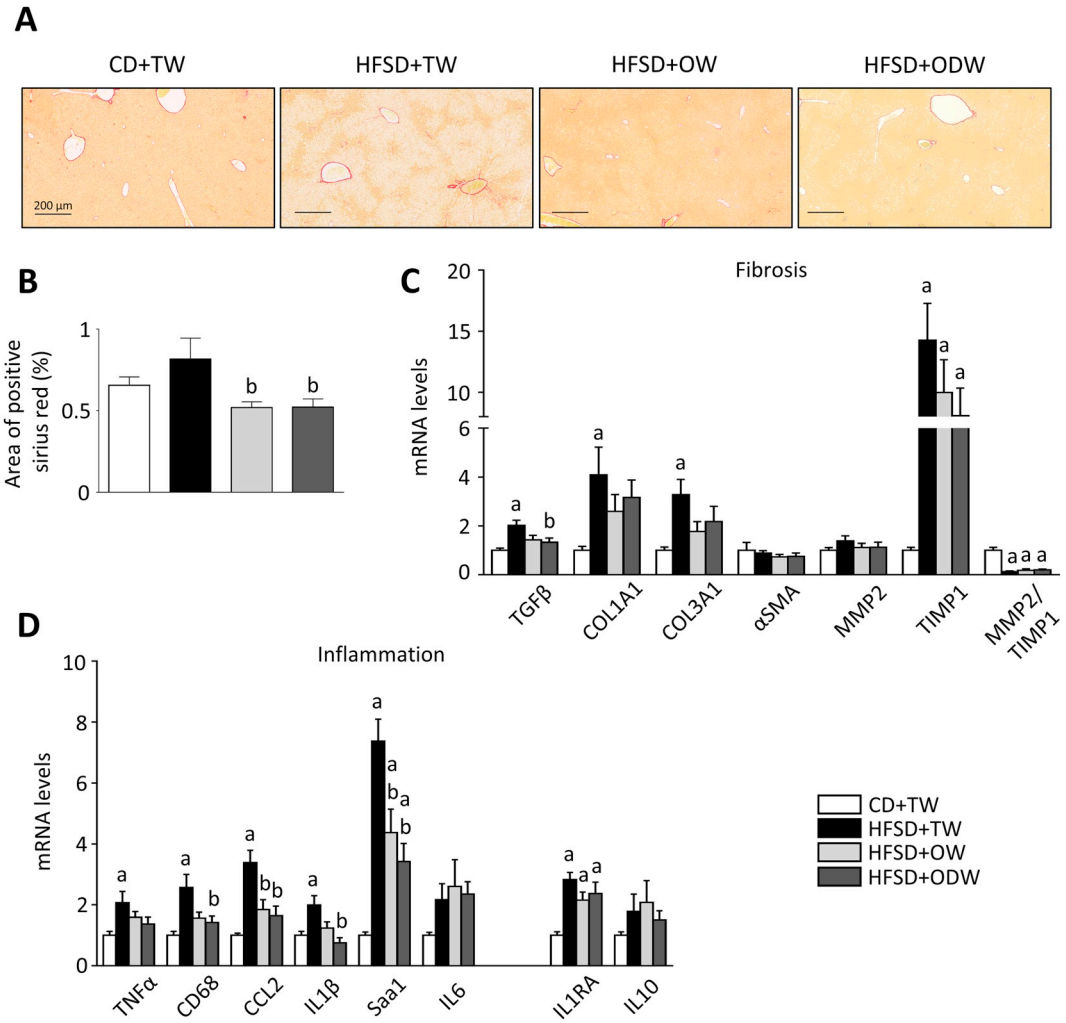

Fig. 3. Effects of modified water on hepatic fibrosis and inflammation. A) Representative picro-sirius red staining of liver sections from each group (bar = 200 µm). B) Quantification of positive Sirius red area (%), n = 10/group. C) mRNA expression level markers of fibrosis: transforming growth factor beta (TGFβ), collagen type I alpha 1 (COL1A1), collagen type III alpha 1 (COL3A1), alpha smooth muscle actin (αSMA), matrix metalloproteinase-2 (MMP2) and TIMP metallopeptidase inhibitor 1 (TIMP1) and MMP2/TIMP1, n = 9/group. D) mRNA expression level of inflammation markers: necrosis factor alpha (TNFα), cluster of differentiation 68 (CD68), chemokine ligand 2 (CCL2), interleukin-1 beta (IL1β), serum amyloid A1 (Saa1), interleukin-6 (IL6) and anti-inflammatory markers: interleukin-1 receptor antagonist (IL1RA) and interleukin-10 (IL10), n = 9/group. Data are mean ± S.E.M. a, vs CD + TW; b, vs HFSD + TW; p < 0.05.

expression levels of pro-inflammatory markers such as tumor necrosis factor alpha (TNFα), cluster of differentiation 68 (CD68), chemokine ligand 2 (CCL2), interleukin-1 beta (IL1β) and serum amyloid A1 (Saa1) were significantly increased in HFSD + TW compared to CD + TW. This suggests a hepatic inflammatory stress in these animals, although the anti-inflammatory interleukin-1 receptor antagonist (IL1RA) mRNA level was also increased. Pro-inflammatory interleukin-6 (IL6) and anti-inflammatory interleukin-10 (IL10) mRNA levels were increased in all HFSD-fed groups compared to CD + TW without significant difference between groups. Interestingly, TNFα, CD68, CCL2, and IL1β mRNA expression levels were lower in HFSD + OW and HFSD + ODW compared to HFDS + TW. Moreover, no significant difference was measured in the expression of these markers in HFSD + OW and HFSD + ODW compared to CD + TW, indicating similar macrophages infiltration in these animals (Fig. 3D).

3.6. ODW intake reduces insulin resistance

Since insulin resistance is part of MetS, whole-body glucose tolerance was investigated with the IPGTT and showed a significant decrease in glucose handling in all HFSD-fed groups compared to CD + TW, as represented by a higher AUC (Fig. 4A). HFSD + ODW mice presented a significant decrease in AUC compared to HFSD + TW and HFSD + OW, albeit still elevated compared to CD + TW. In addition, HFSD-fed mice presented higher fasting glycaemia and insulinemia compared to CD + TW (Table 4). Thus, the homeostatic model assessment of insulin resistance (HOMA-IR) was increased in all HFSD-fed mice compared to CD + TW (Fig. 4B). However, HOMA-IR was significantly decreased in HFSD + ODW mice and near significance with HFSD + TW, illustrating that HFSD + ODW mice presented an improved insulin sensitivity compared to other HFSD-fed mice. Since skeletal muscles are major regulators of insulin-stimulated glucose uptake, we investigated muscle insulin resistance with the P-Akt/Akt *ratio*. We found a significant decrease in P-Akt/Akt *ratio* in HFSD + TW and HFSD + OW as compared to CD + TW. However, P-Akt/Akt *ratio* was increased in HFSD + ODW compared to other HFSD-fed groups, with no significant difference compared to CD + TW (Fig. 4C and D), illustrating that ODW improved insulin sensitivity at the whole body and muscle tissue levels.

3.7. OW and ODW intakes reduce hepatic glycogen content

Since the liver has the ability to store glucose in the form of glycogen, which is associated with insulin sensitivity, we measured glycogen content in liver. Hepatic glycogen content was similar in CD + TW and HFSD + TW mice, but significantly decreased in HFSD + OW and HFSD + ODW (Fig. 4E). As shown in Table 3, HFSD feeding induced an increase in liver weight compared to chow. Among HFSD-fed mice, livers from HFSD + ODW mice weighted significantly less than the ones from HFSD + TW. When hepatic glycogen content was reported to liver weight, a significant decrease was observed in all HFSD-fed groups, suggesting a decrease in hepatic insulin sensitivity.

4. Discussion

This prospective study aimed to investigate the impact of daily consumption of biocompatible OW or ODW, compared to TW, on the development of MetS features in mice fed a HFSD.

Our results first demonstrate that in mice fed an enriched fat and sucrose diet, the daily intake of OW, and thus, the reverse osmosis filtration of TW, induces striking benefits on MetS features by decreasing hepatic fibrosis and inflammation and counteracting hepatic steatosis and serum dyslipidemia. Interestingly, several studies have demonstrated that similar effects, generally achieved by multiple pharmaceutical approaches, could also be obtained by global non-pharmaceutical nutritional interventions such as the intake of high bicarbonated mineral water or following high water intake (Naumann et al., 2017).

Compared to TW, the OW generated by reverse osmosis is supposed to be exempt of pollutant and shares physico-chemical properties of a low mineralized water (Table 1). The daily consumption of low mineral content water is claimed to be safe for everyday use for all age groups,

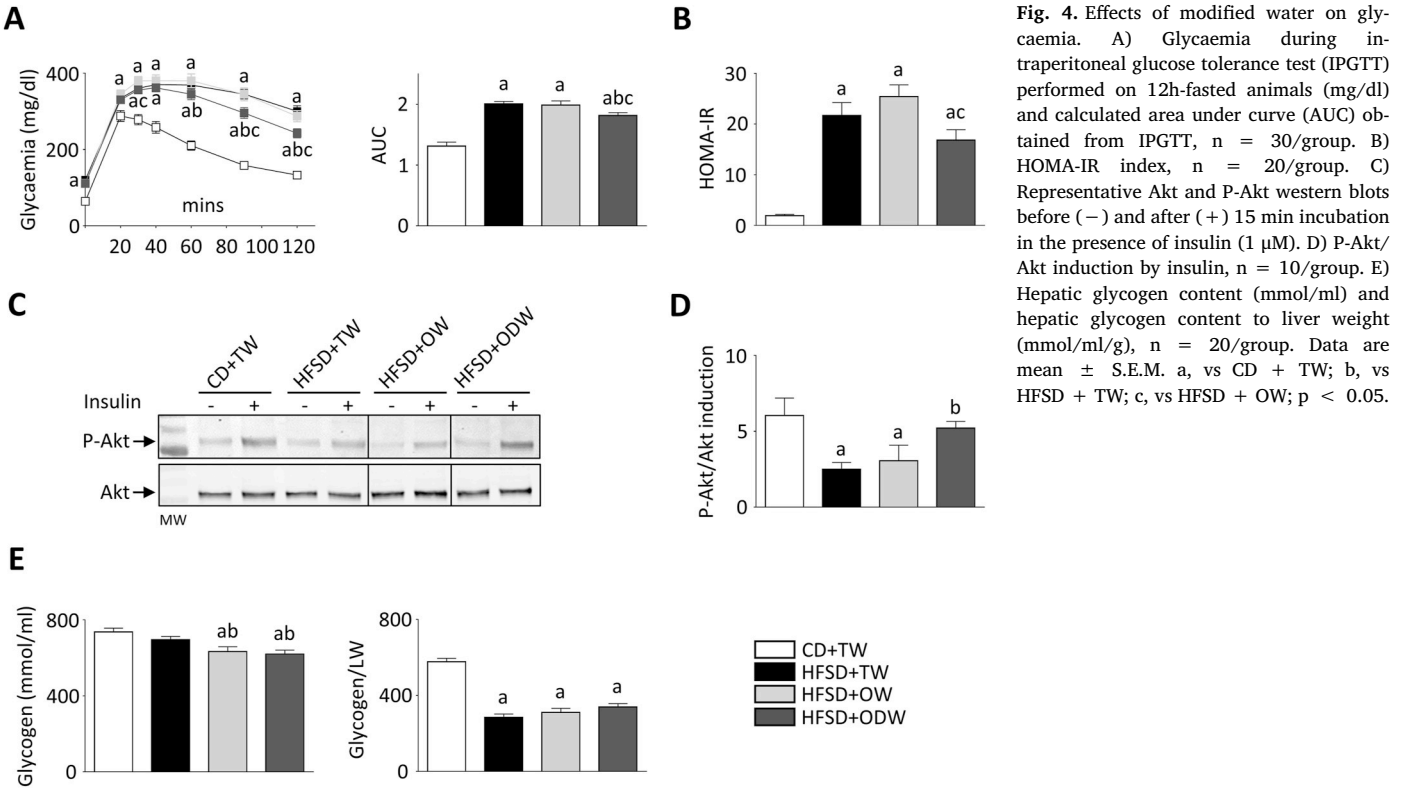

Fig. 4. Effects of modified water on glycaemia. A) Glycaemia during intraperitoneal glucose tolerance test (IPGTT) performed on 12h-fasted animals (mg/dl) and calculated area under curve (AUC) obtained from IPGTT, n = 30/group. B) HOMA-IR index, n = 20/group. C) Representative Akt and P-Akt western blots before (−) and after (+) 15 min incubation in the presence of insulin (1 μM). D) P-Akt/Akt induction by insulin, n = 10/group. E) Hepatic glycogen content (mmol/ml) and hepatic glycogen content to liver weight (mmol/ml/g), n = 20/group. Data are mean ± S.E.M. a, vs CD + TW; b, vs HFSD + TW; c, vs HFSD + OW; $p < 0.05$.

and advised in many health conditions but not specifically in the treatment of MetS. Instead, the intake of high bicarbonated water with moderate alkalinity and specific osmotic properties, was shown to be beneficial for dyslipidemia and glucose metabolism (Schoppen et al., 2004; Toxqui and Vaquero, 2016), suggesting that the beneficial effects observed in our study by the daily intake of OW are not related to specific or high mineral amounts. Interestingly, it is accepted that high mineral and heavy metal concentration in water can lead to interactions between minerals such as co-precipitation, the formation of insoluble complexes, as well as competitive interactions, responsible of a decrease in water and solutes absorption in the intestine. Considering the lower minerals concentration of OW and ODW, compared to TW, it is one possibility that the positive effects observed in our study could be in part mediated by better intestine water and ions bioavailability and absorption, overall positively affecting physiological processes such as enzymatic functions, nutrients assimilation or toxins and waste elimination.

High water intake, by decreasing circulating AVP concentrations, was also associated to a decrease in MetS features such as hepatic steatosis and dyslipidemia in obese rats, as well as T2DM risk in patients (Carroll et al., 2015; Enhörning et al., 2018; Taveau et al., 2015). Interestingly, our results demonstrate that water intake in the OW group is significantly greater than in TW and ODW HFSD-fed mice. Thus, one can hypothesize that the beneficial effects observed on hepatic steatosis and dyslipidemia, in mice consuming OW, may be in part attributed to a decrease in AVP concentrations. However, low circulating AVP is also associated with a decrease in hepatic gluconeogenesis and glycogenolysis, with reduced plasma glucose, lower risks of hyperglycemia, and increased hepatic glycogen content (Carroll et al., 2015; Enhörning et al., 2018; Taveau et al., 2015). In our study, all HFSD-fed mice presented high and similar fasting glycaemia and mice with OW and ODW intake also presented decreased hepatic glycogen content, compared to TW. These observations suggest that low circulating AVP in HFSD-fed mice consuming OW may have dissociated effects and play a part in decreasing hepatic steatosis and dyslipidemia independently of hyperglycemia and hepatic glycogen content, as previously observed in other murine models (Moon et al., 2012; Wendel et al., 2010).

Lastly, it is also now well documented that our drinking water resources are being contaminated by a mixture of pollutants (Benotti et al., 2009; Wee and Aris, 2017), such as metabolic disruptors or environmental obesogenic factors. Those are known to interfere with pro-inflammatory mechanisms, which promote metabolic alterations in the obesity epidemic (Hotamisligil, 2006). These pollutants have been correlated with an increased risk of metabolic disorders and to the rising in obesity, NAFLD, T2DM, in humans (Casals-Casas and Desvergne, 2011; Foulds et al., 2017). More specifically, numerous animal studies have demonstrated that endocrine-disrupting chemicals exposure worsens metabolic consequences, such as inflammation, fibrosis, lipid and glucose metabolism dysfunctions, as well as hepatic steatosis (Duval et al., 2017; Seth et al., 2013; Wahlang et al., 2014; Wei et al., 2014; Yu et al., 2018), and can be a risk factor for MetS features when associated with a high energy diet. Since the three most frequent causes for hepatic steatosis are alcohol, MetS, and environmental toxicants, in a free alcohol context such as in our study, the hepatic steatosis observed in HFSD + TW mice, appears to be a pathologic liver response to inflammation, diet composition and pollutant exposure (Minihane et al., 2015). Considering that reverse osmosis is an effective way to completely remove pollutant molecules from TW, the beneficial effects observed on MetS features in HFSD mice consuming biocompatible reverse osmosed water (OW and ODW) are likely to be associated to the withdrawal of these molecules from TW. Moreover, our results suggest that the effects obtained with OW and ODW intakes could be mediated through changes in gut microbiota composition. Indeed, altered gut microbiota composition is associated to nutrient and mineral absorption, inflammation, MetS, obesity and diabetes (Caesar et al., 2010; He and Shi, 2017; Ley et al., 2006; Tremaroli and Bäckhed, 2012), and it has been shown to be modified by different type of drinking water (Dias et al., 2018), as well as endocrine-disrupting chemicals in drinking water (Zhu et al., 2019).

The intake of OW and ODW showed similar benefits on hepatic steatosis, fibrosis, glycogen content, and serum dyslipidemia. However, the dynamization process applied on the ODW induced further improvement in glucose tolerance and muscle insulin sensitivity. Indeed, HFSD-fed mice receiving ODW were characterized by decreased glucose concentrations and glucose AUC during IPGTT, improved insulin sensitivity in skeletal muscle, and decreased basal metabolism. Interestingly, since liver insulin sensitivity is not further improved by ODW compared to OW, this may indicate a tissue-specific effect on muscles, a tissue highly sensitive to nutritional intervention and crucial in insulin sensitivity.

Due to the technical design of the dynamization process, defining how ODW intake positively affect glycaemia in our study can only be hypothesized. Indeed, this process involves the application of an electric current, and thus of magnetic and electromagnetic fields, on water through a silver electrode. It has been known for decades that water is bipolar and diamagnetic and that each of these applied physical factor exerts multiple, complex and sometimes opposite effects on water physicochemical properties and on water structure and composition (Bramwell, 1999; Chibowski and Szcześ, 2018; Vallée et al., 2005; Wang et al., 2018; Yamashita et al., 2003). However, these effects still remain ambiguous and controversial and the mechanisms by which modified water molecules affect biological system and/or function still remain largely debated.

Interestingly, another study demonstrated that magnetized water supplementation improved diabetes through the reduction of blood glucose level in streptozotocin-treated rats (Lee and Kang, 2013). This article supports the notion that magnetized water may improve glucose metabolism. According to the authors, these effects could be related to the specific hexagonal structure of magnetized water (Lee and Kang, 2013). Other extensive works reported that any electromagnetic energy applied on water induces the formation of “exclusion zone” (EZ) water (Zheng et al., 2006). Even if still debated, EZ water has been defined as a hexagonal ordered liquid crystal water, with similar properties to interfacial or biological water in the cell. These observations, together with the facts that water is the main cellular constituent and that proper hydration plays a vital role in cell function, led to the hypothesis that increasing EZ water within the cell would promote health benefits (Sharma et al., 2018). Since the water dynamization process used in our study involves the application of electromagnetic energy on water, one can hypothesize that it could generate EZ water. Thus, the daily intake of ODW could result, at the molecular level, in better conditions for biological activities of macromolecules such as proteins and nucleic acids, and improve protein folding or enzymatic functions (Ball, 2017; Chaplin, 2006). This should result at the cellular level, to improvement in cell wall permeability, increased bioavailability and hydration of water compounds, nutrients assimilation, or toxins and cellular waste elimination.

It must also be considered that the current injected in the electrode may induce water electrolysis and a possible enrichment in H_2 molecules with ROS scavenging and antidiabetic properties, as described for biocompatible ERW (Shirahata et al., 2012). Electrolysis may also induce the release of bio-available colloidal silver ions or silver particles in water, from the silver electrode. Moreover, the properties of these colloidal may be affected by the applied electromagnetic field or participate in EZ water formation. In therapeutics using trace element, colloidal silver is used for its anti-inflammatory properties and thus, could indirectly contribute to the improvement of glucose metabolism observed with ODW intake, by counteracting inflammation as observed with magnesium (Dibaba et al., 2014). More interestingly, the use of electrodes made of different minerals such as zinc, chromium, magnesium or vanadium could lead to the generation of enriched-colloidal

water specifically alleviating insulin resistance (Kim et al., 2018). Overall, more detailed and in-depth experiments are clearly warranted to define the mechanisms involved in the positive effects of ODW intake on glucose metabolism.

In conclusion, this work demonstrates that in mice fed a HFSD, the daily intake of biocompatible modified water such as OW and ODW, prevents some major features of MetS, compared to TW, and that water dynamization process can positively affect glucose metabolism. It highlights the possible deleterious effects of daily intake of TW when associated to a high energy diet and provides insights into an interplay between environmental contaminants contained in TW and diet composition. Our data also emphasize the need for further studies to characterize the mechanisms of action of biocompatible dynamized water in exerting biological effects on obese insulin-resistant human. This could lead to the development of global non-pharmaceutical, cost-effective and safe nutritional intervention for the prevention of cardiometabolic risk factors associated to MetS in obese insulin-resistant human.

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CRediT authorship contribution statement

Karen Lambert: Conceptualization, Methodology, Investigation, Validation, Formal analysis. **Claire Gondeau:** Methodology, Investigation, Validation, Formal analysis, Writing - review & editing. **Philippe Briolotti:** Investigation, Formal analysis. **Valérie Scheuermann:** Investigation, Formal analysis. **Martine Daujat-Chavanieu:** Formal analysis, Validation, Writing - review & editing. **Franck Aimond:** Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Writing - original draft, Supervision.

Declaration of competing interest

None.

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