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Effects of ethanol or ethylene glycol exposure on PPARγ and aromatase expression in adipose tissue

Jacob Ardenkjær-Skinnerup [a,b], Daniel Saar [c], Sofie Christiansen [a], Terje Svingen [a], Niels Hadrup [a,b], Kristy A. Brown [d,e], Brice Emanuelli [f], Birthe B. Kragelund [c], Gitte Ravn-Haren [a,*], Ulla Vogel [a,b,**]

[a] The National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark
[b] The National Research Centre for the Working Environment, Copenhagen Ø, Denmark
[c] REPIN and Structural Biology and NMR Laboratory, Department of Biology, University of Copenhagen, Copenhagen N, Denmark
[d] Department of Medicine, Weill Cornell Medicine, New York, NY, USA
[e] Department of Cell Biology and Physiology, University of Kansas Medical Center, Kansas City, KS, USA
[f] The Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Copenhagen N, Denmark

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ABSTRACT

The estrogen-synthesizing enzyme aromatase is expressed in adipose tissue where it controls the local concentration of estrogen. It has been suggested that the organic solvents ethanol and ethylene glycol can induce estrogen synthesis by inhibiting PPARγ activity. Since elevated estrogen synthesis in adipose tissue is a risk factor for breast cancer development, it is of interest to further characterize the mechanisms regulating aromatase expression. Here, we explored the mechanisms by which ethanol and ethylene glycol modulate aromatase mRNA expression and the ultimate conversion of androgens into estrogens.

NMR spectroscopy revealed that ethanol and ethylene glycol influence the active state of PPARγ. An inhibitory effect on PPARγ was confirmed by adipogenesis assays and PPARγ target gene expression analysis in adipocytes. However, only ethanol increased aromatase mRNA in differentiated human adipocytes. In contrast, ethylene glycol downregulated aromatase in a PPARγ-independent manner. An animal study using female Wistar rats was conducted to assess the acute effects of ethanol and ethylene glycol on aromatase expression in adipose tissue within a physiological context. No changes in aromatase or PPARγ target gene (*Adipoq* and *Fabp4*) levels were observed in adipose tissue or ovary in response to the chemical exposures, suggesting an absence of acute PPARγ-mediated effects in these organs.

The results suggest that ethanol and ethylene glycol are weak PPARγ antagonists in mouse and human adipocytes as well as in cell-free NMR spectroscopy. Both compounds seem to affect adipocyte aromatase expression *in vitro*, where ethanol increased aromatase expression PPARγ-dependently and ethylene glycol decreased aromatase expression independently of PPARγ. No acute effects on aromatase expression or PPARγ activity were observed in adipose tissue or ovary in rats in this study design.

1. Introduction

Breast cancer remains a significant global health concern with continued rise in incidence rates. Common risk factors include increasing age, hormone therapy, obesity, and sedentary behavior [1]. Extensive research efforts have been dedicated to explaining the interplay between environmental factors, hormonal imbalances, and breast cancer development [2]. Among the lifestyle factors, alcohol consumption stands out as a major risk factor. Specifically, ethanol has been associated with increased development of estrogen receptor-positive tumors, potentially mediated by an increase in estrogen levels [3]. Thus, one drink per day (10 g alcohol) was associated with 4.2 % (95 % CI: 2.7–5.8 %) increased risk of breast cancer in the EPIC cohort [4].

Like exposure to ethanol, occupational exposure to other organic

* Corresponding author. National Research Centre for the Working Environment, Copenhagen Ø, Denmark.
** Corresponding author. National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark.
E-mail addresses: girh@food.dtu.dk (G. Ravn-Haren), ubv@nfa.dk (U. Vogel).

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solvents has been linked to an increased risk of breast cancer [5]. Although there is consistent evidence linking breast cancer to alcohol consumption, the mechanism-of-action for carcinogenesis induced by ethanol, as well as other organic solvents, is not fully understood. Ethanol and its metabolite acetaldehyde are both classified group 1 carcinogens according to IARC [6], but it has been suggested that it is ethanol rather than acetaldehyde that is the carcinogenic substance in postmenopausal breast cancer [7]. Another highly relevant alcohol is ethylene glycol, a high-production-volume industrial compound present in numerous products, such as heat-transfer fluids, detergents, and inks [8]. Studies suggest that ethanol and ethylene glycol may affect breast cancer risk via a mechanism involving the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) [9,10]. Thus, the functional polymorphism *PPARG2* Pro12Ala, where a proline is substituted by an alanine in the N-terminal sequence of PPARγ2, is a determinant of alcohol-related breast cancer in the prospective epidemiological Diet, Cancer and Health study [9,11]. Both ethanol and ethylene glycol have been reported to inhibit PPARγ activity in HEK293 cells [9,10] and PPARγ expression in rodent kidney [12,13] and adipose tissue [14,15].

PPARγ is a ligand-activated transcription factor abundantly expressed in adipose tissue where it regulates adipogenesis, lipid metabolism, and insulin sensitivity [16]. There are two main isoforms, PPARγ1 and PPARγ2, the latter being preferentially expressed in adipocytes and being a stronger inducer of adipogenesis [17]. *In vitro* studies suggest that PPARγ activation protects against breast cancer via repression of aromatase (*CYP19A1*) [18,19]. In contrast, PPARγ can be inhibited by environmental antagonists [20], which leads to upregulation of aromatase in human adipose tissue culture [21]. Since ethanol and ethylene glycol have been demonstrated to inhibit PPARγ signaling, they may promote breast carcinogenesis through a mechanism involving upregulation of aromatase in adipose tissue.

This study aimed to elucidate the impact of ethanol and ethylene glycol on aromatase expression in adipose tissue by employing *in vitro* and *in vivo* methods. First, interactions with the PPARγ ligand binding domain (LBD) were studied *in vitro* to uncover whether observed downstream effects were directly or indirectly related to PPARγ activity. Effects on adipogenesis and acute aromatase regulation were then studied in mouse and human adipose stromal cell (ASC) lines, respectively. Finally, the expression of aromatase and aromatase-associated cytokines was investigated in rat adipose tissue in response to short-term exposure to ethanol and ethylene glycol, using GW9662 as a control antagonist of PPARγ.

2. Materials and methods

2.1. Animal study

Twenty-four female Wistar (Han) rats (purchased from Charles River Laboratories, Sandhofer Weg 7, Sulzfeld, Germany; distributed by SCANBUR, Denmark), age 7–8 weeks, with mean body weight of 180 g were received at the animal facilities (BioFacility) at DTU, Lyngby, Denmark. The animals were housed in high-temperature polysulfone (PSU) cages with Tapvei wooden shelters. The cages were placed in ScanTainers (ventilated cabinets from SCANBUR) with controlled environmental conditions: 12 h light (9.00–21.00 h), 12 h dark (21.00–9.00 h) cycle, humidity 55 % ± 5 %, temperature 22 °C ± 10 °C, and ventilation changing air 50–60 times per hour. Animals were fed Altromin 1314 (soy and alfalfa free) and tap water (BPA-free bottles 84ACBT0402PFS polysulfone 400 mL w/ring square, SCANBUR, Denmark) *ad libitum*.

The animals were pseudo-randomly distributed (based on body weight) into one control group and three experimental groups ($n = 6$), in which the animals were exposed to either GW9662 (M6191, Sigma-Aldrich), ethanol (Navimer Alcohol Pur 96 %), or ethylene glycol (324558, Sigma-Aldrich). Rats were administered ethanol or ethylene glycol via drinking water at concentrations of 10 % or 0.75 %, respectively, while GW9662 (2 mg/day) was delivered in a semi-solid vehicle consisting of 1 g hazelnut chocolate cream (Nutella®, Ferrero) on a 0.8 g Marie biscuit (Salling Group) [22]. The daily intake of GW9662, ethanol, and ethylene glycol were 10 mg/kg bw, 14 g/kg bw, and 1.3 g/kg bw, respectively, and all groups received the vehicle (with or without GW9662). The vehicle provided additional fatty acids to elevate the activity and expression level of PPARγ for better detection of inhibitory effects on PPARγ. The chemical doses were selected based on studies showing effects on aromatase or PPARγ expression in response to similar doses of orally administered ethanol [23], ethylene glycol [12,13], or GW9662 [24]. Rats were housed in pairs and temporarily relocated to separate cages during delivery of vehicle with or without GW9662.

The rats received vehicle (hazelnut chocolate cream without chemical) for five days, followed by a two-day weekend break, to acclimatize them to the taste of the vehicle before chemical exposure. Then rats were exposed to chemicals for two days, and finally euthanized (anesthetized with CO_2/O_2 and decapitated) on the tenth day. Ethanol and ethylene glycol were accessible up until euthanasia. The exposure period of two days was selected to study the acute effect of the chemicals on *PPARG* and aromatase and to minimize the risk of renal toxicity induced by ethylene glycol, which has been shown to cause kidney stones after long-term exposure to a dose of 0.75–1.0 % in the drinking water [12,13]. Ovaries, trimmed from the fat pad, as well as mammary and visceral adipose tissues were collected and immediately immersed in RNA*later* (AM7021, Invitrogen).

The animal experiment was conducted at the DTU BioFacility, DTU Health Tech, Technical University of Denmark (DTU, Lyngby). Ethical approval was obtained from the Danish Animal Experiments Inspectorate (Council for Animal Experimentation, authorization number 2020-15-0201-00570), and the experiment was monitored by the Animal Welfare Committee of the Technical University of Denmark. For this study, we got an extension of the permit to also cover dosing of GW9662 in the hazelnut chocolate cream vehicle.

2.2. Cell culture

Primary cells were isolated from human adipose tissue and cultured as described previously [21]. The cells were isolated from adipose tissue obtained from patients undergoing mastectomy, abdominoplasty, or reduction mammoplasty at Weill Cornell Medicine (under IRB-approved protocol #20–01021391). Primary cells were cultured in F-12 medium (10-080-CV, Corning) containing 10 % fetal bovine serum (FBS; 35-010-CV, Corning) and 1 % penicillin-streptomycin solution (15140122, Gibco). The human A41 ASC line (hTERT A41hWAT-SVF) [25] and the mouse C3H10T1/2 mesenchymal stem cell line (CCL-226, ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM; 41965-039, Gibco) containing 10 % FBS (F7524, Sigma-Aldrich) and 1 % penicillin-streptomycin solution (15070063, Gibco). All cells were cultured in humidified incubators at 37 °C and 5 % CO_2. Culture medium was changed every 2 or 3 days.

Undifferentiated or 12-day differentiated A41 cells were exposed to 1 % ethylene glycol (99.8 % purity) or 1 % absolute ethanol (≥99.5 % purity) for 24 h. In the differentiation experiments, primary human ASCs or C3H10T1/2 cells were exposed throughout differentiation at the indicated concentrations.

2.3. Adipocyte differentiation

Primary human ASCs, A41 cells, and C3H10T1/2 cells were induced to differentiate when they were 100 % confluent. Primary human ASCs were washed twice with phosphate-buffered saline (PBS) and differentiated for 12 days using serum-free culture medium containing 0.1 or 2 μM rosiglitazone (day 0–4), 0.25 μM dexamethasone (day 0–6), 500 μM IBMX (day 0–6), 20 nM insulin, 0.2 nM triiodothyronine (T_3), 33 μM biotin, 17 μM pantothenic acid, 0.1 μM transferrin, and 10 μg/mL

cortisol (all from Sigma-Aldrich).

A41 cells were differentiated in serum-containing culture medium supplemented with 1 μM rosiglitazone, 0.1 μM dexamethasone, 500 μM IBMX, 500 nM insulin, 2 nM T_3, 33 μM biotin, and 17 μM pantothenic acid. C3H10T1/2 cells were differentiated by adding 0.1 μM rosiglitazone, 0.2 μM dexamethasone (day 0–2), 100 μM IBMX (day 0–2), and 4 nM insulin (day 0–4) to the serum-containing culture medium. Adipogenic medium was changed every 2, 2, or 3 days for primary ASCs, C3H10T1/2, and A41 cells, respectively.

In experiments, where primary ASCs or C3H10T1/2 cells were exposed to chemicals during differentiation, the chemicals were added each time differentiation medium was renewed. Mature A41 cells, used for acute chemical exposure, were differentiated for 12 days, and returned to regular growth medium for 2 days before 24 h chemical exposure.

2.4. Lipid staining and quantification

Primary human ASCs were differentiated in transparent 96-well plates. On day 12 of differentiation, they were washed in PBS and fixed with 4 % formaldehyde (252549, Sigma-Aldrich) in PBS for 30 min at room temperature. Then cells were washed twice with water and incubated with 60 % isopropanol for 5 min, followed by further incubation with sterile filtered 60 % Oil Red O (O0625, Sigma-Aldrich) solution for 20 min. Cells were washed 3 times with water and then viewed under the microscope. Stained lipids were quantified by washing 3 times with 60 % isopropanol for 5 min, and then extracting the Oil Red O stain with 50 μL 100 % isopropanol for 20 min. Finally, 40 μL of the extracted Oil Red O was transferred to a 384 well plate. Absorbance was read at 518 nm, and 100 % isopropanol was used as background control.

2.5. Gene expression analysis

Cells (A41 and C3H10T1/2) were washed in PBS before lysis. Cultured cells and ovaries were lysed with Buffer RLT (Qiagen) containing 1 % β-mercaptoethanol, and RNA was extracted using RNeasy Kit (Qiagen). Adipose tissue samples were lysed with QIAzol (Qiagen), and RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen). For cell lines, cDNA synthesis from 1 μg RNA was performed using iScript cDNA Synthesis Kit (1708891, Bio-Rad), and quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (600882, Agilent) and the CFX384 Real-Time PCR Detection System (Bio-Rad). Primers were purchased from TAG Copenhagen and are shown in Table 1. For tissue samples, Omniscript RT Kit (205113, Qiagen), SUPERase-In RNase Inhibitor (AM2694, Invitrogen), and Random Primer Mix (S1330, New England Biolabs) were used for cDNA synthesis from 2 μg RNA. TaqMan Fast Advanced Master Mix (4444557, Applied Biosystems) and the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) were applied for qPCR. TaqMan assays (4331182, Applied Biosystems) were used for rat *Rps18* (Rn01428913_gH), *Cyp19a1* (Rn00567222_m1), *Pparg* (Rn00440945_m1), *Fabp4* (Rn04219585_m1), *Adipoq* (Rn00595250_m1), *Lep* (Rn00565158_m1), and *Il6* (Rn01410330_m1). Each biological sample was measured in technical triplicates, and the $2^{-\Delta\Delta Ct}$ method was used for relative quantification.

2.6. Protein production

Human PPARγ LBD cDNA (residues 231 to 505) with an N-terminal hexahistidine- and SUMO-tag (H_6-SUMO) was cloned into a modified pET24a vector (Twist). Protein production was performed in *E. coli* BL21 (-DE3) cells (New England BioLabs, Frankfurt, Germany) in auto-induction minimal medium [26], with $^{15}NH_4Cl$ as a nitrogen source for isotope labeling. The temperature was changed at OD_{600} of 0.8 from 37 °C to 18 °C, and protein production was allowed to proceed for 24 h. Cells were harvested by centrifugation at 5000×*g* for 20 min. For purification, pellets were resuspended in lysis buffer (20 mM imidazole, 50 mM Tris pH 8, 200 mM NaCl, 10 % (v/v) glycerol). All purification buffers contained 5 mM β-mercaptoethanol. Cells were lysed with a cell disrupter (Constant Systems Ltd., Daventry, UK) at 25 kpsi and the lysate was cleared by centrifugation at 20,000×*g* for 45 min. The supernatant, pre-equilibrated with lysis buffer, was twice passed over 5 mL Ni-NTA resin (Qiagen, Hilden, Germany). The three wash steps were done with lysis buffer first, then with lysis buffer containing 1 M NaCl, and finally with lysis buffer again. For elution of bound proteins, lysis buffer with 500 mM imidazole was used. The protein was cleaved overnight at 4 °C using ULP1-protease (in-house production) under dialysis into 40 mM Tris, pH 8, 10 % glycerol, 200 mM NaCl, and 5 mM β-mercaptoethanol. The His_6-SUMO tag was removed by passing over the Ni-NTA column again. Purification continued by ion exchange chromatography using a HiTrap QFF 5 mL column (Cytiva) and an ÄKTA pure 25 chromatography system (GE Healthcare, Munich, Germany) and size exclusion chromatography using a Superdex 200 Increase 10/300 GL (Sigma-Aldrich). Ion exchange buffers were 25 mM bis-Tris pH 7.4, with the elution buffer containing 1 M NaCl in addition. The buffer for size exclusion contained 40 mM Tris pH 8 and 500 mM NaCl.

2.7. Nuclear magnetic resonance (NMR) spectroscopy

NMR samples were kept at room temperature and contained 80 μM ^{15}N PPARγ LBD in PBS buffer, pH 7.3, 137 mM NaCl, 10 % D_2O (v/v). As a reference, 0.7 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added. Ethanol or ethylene glycol was added to a final concentration of 3 % (v/v). ^{15}N-HSQC NMR spectra were recorded at 298 K on a Bruker AVANCE III 750-MHz (^1H) spectrometer equipped with a cryogenic probe. Free induction decays were transformed and visualized in Topspin (Bruker Biospin), and subsequently analyzed using the CcpNmr Analysis software [27]. Proton chemical shifts were internally referenced to DSS at 0.00 ppm with heteronuclei referenced by relative gyromagnetic ratios. Assignments of peaks of PPARγ LBD were exported from BMRB [28] and transferred from an assignment by Hughes et al., 2012 [29]. Intensities were internally normalized to the E235 peak of each spectrum, which was the most intense peak in every condition.

Table 1
Primers used for qPCR using SYBR Green.

Gene	Species	Sequence **(forward)**	Sequence **(reverse)**
RPL32	Human	5′-CAGGGTTCGTAGAAGATTCAAGGG-3′	5′-CTTGGAGGAAACATTGTGAGCGATC-3′
CYP19A1	Human	5′-TTGACCCTTCTGCGTCGTGT-3′	5′-AGGAGAGCTTGCCATGCATCA-3′
ADIPOQ	Human	5′-GCAGTCTGTGGTTCTGATTCC-3′	5′-CATGACCGGGCAGAGCTAAT-3′
FASN	Human	5′-TACAACATCGACACCAGCTC-3′	5′-CGTCTTCCACACTATGCTCA-3′
Cyp19a1	Rat	5′-CGCAGAGTATCCGGAGGTGG-3′	5′-CTGATACCGCAGGCTCTCGT-3′
Rn18s	Mouse	5′-AGTCCCTGCCCTTTGTACACA-3′	5′-GATCCGAGGGCCTCACTAAAC-3′
Pparg2	Mouse	5′-GCATGGTGCCTTCGCTGA-3′	5′-TGGCATCTCTGTGTCAACCATG-3′
Adipoq	Mouse	5′-GATGGCACTCCTGGAGAGAA-3′	5′-TCTCCAGGCTCTCCTTTCCT-3′
Fabp4	Mouse	5′-CTGGGCGTGGAATTCGAT-3′	5′-GCTCTTCACCTTCCTGTCGTCT-3′
Slc2a4	Mouse	5′-GTGACTGGAACACTGGTCCTA-3′	5′-CCAGCCACGTTGCATTGTAG-3′

2.8. Statistical analysis

Statistical significance was assessed by Dunnett's multiple comparisons test or two-way analysis of variance (ANOVA) using GraphPad Prism 10.1.2, depending on the number of variables. When there were more than two levels within a variable of the two-way ANOVA, Dunnett's test for multiple comparisons was applied for each level of the variable or for the means of the levels within the variable. Data was normalized to the sum of values within each experiment, and the control group was set to 1. Differences between groups were considered significant if $p \leq 0.05$, and data were presented as means and standard errors of the mean (SEM).

3. Results

3.1. Ethanol and ethylene glycol affect the active state of PPARγ LBD

NMR spectroscopy was used to verify previously reported inactivation of PPARγ by ethanol and ethylene glycol [9,10]. An activity control was established in a previous study by recording ^{15}N-HSQCs of PPARγ LBD bound to the known agonist rosiglitazone in the presence or absence of the antagonist GW9662 [21]. These spectra were used as negative and positive controls for activity states of PPARγ LBD, respectively (Fig. 1A and B) [21]. The number and NMR peak intensity of annotatable signals, especially around the ligand-binding pocket and in helix 12 of PPARγ LBD, were used as an indication for activity (Fig. 1F). The rosiglitazone-bound active state featured 208 peaks that could be assigned, corresponding to ~75 % of the residues of the LBD (Fig. 1E) [21]. Addition of GW9662 to rosiglitazone-bound PPARγ LBD reverted the number of assignable peaks to ~40 % of the possible NMR signals of the LBD, stabilizing the inactive state (Fig. 1B and E) [21]. Adding ethanol or ethylene glycol to rosiglitazone-bound PPARγ LBD led to changes in chemical shifts and NMR peak intensities (Fig. 1A, C, 1D), but far from as pronounced as for GW9662, resulting in ~74 % and ~75 % of the residues being accounted for, respectively (Fig. 1E). These peaks were in general less intense compared to PPARγ LBD without addition of ethanol and ethylene glycol. Neither ethanol nor ethylene glycol had any effect on the basal state of PPARγ LBD (Supplemental Fig. S1), where all peaks globally shifted slightly and in the same direction, likely caused by an effect from the changed solution rather than an effect specifically on the PPARγ LBD. This suggests that ethanol and ethylene glycol to some extent influence the active state of PPARγ LBD, either by interacting directly with the LBD to affect rosiglitazone binding or by extracting rosiglitazone from the LBD, with the largest effect obtained with ethanol. The full mechanism cannot be elucidated based on these data.

3.2. Effect of ethanol and ethylene glycol on adipogenesis

To determine if ethanol or ethylene glycol influenced adipogenesis, mouse C3H10T1/2 cells were exposed to ethanol or ethylene glycol during differentiation, and mRNA levels of four adipocyte markers (*Pparg2*, *Adipoq*, *Fabp4*, and *Slc2a4*) were measured. Exposure to 1 % ethanol during differentiation induced a modest reduction in adipocyte marker mRNA abundance (Fig. 2A). A concentration-dependent effect was also observed in response to ethylene glycol (0.3 and 1 %), which reduced mRNA markers by about 50 % at a concentration of 1 % (Fig. 2B).

To determine if the effect observed in mouse cells could also be found in human cells, primary human ASCs were differentiated in the presence

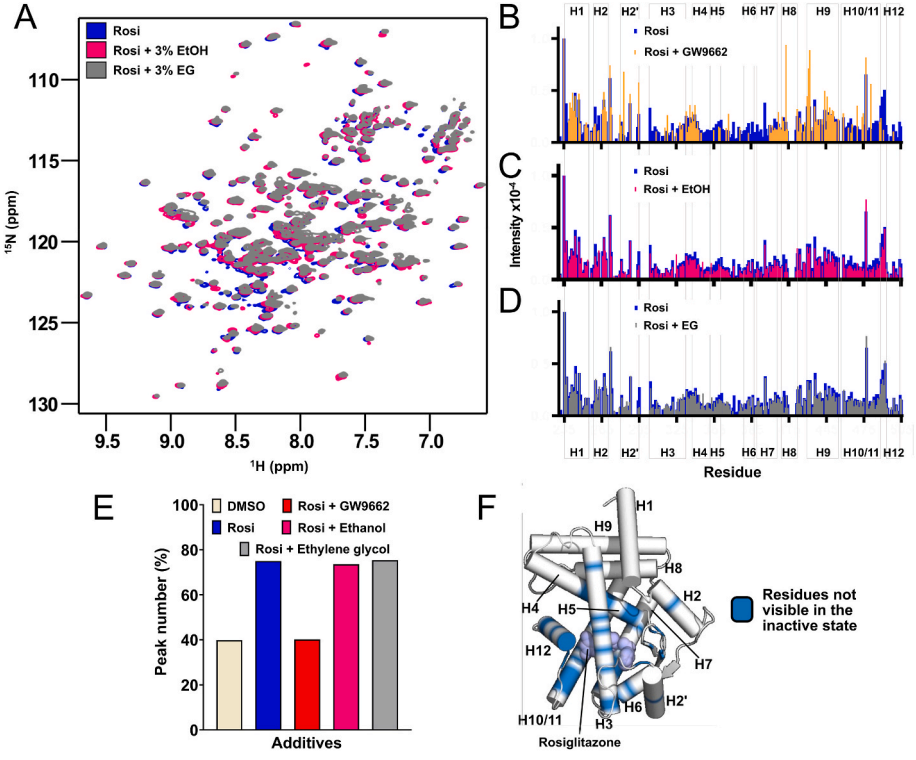

Fig. 1. Direct interactions of ethanol and ethylene glycol with PPARγ LBD in the presence of rosiglitazone. NMR spectroscopy was performed using PPARγ LBD and different chemical compounds. The data for rosiglitazone alone or together with GW9662 were previously reported [21]. (**A**) ^{15}N-HSQCs of the PPARγ LBD together with rosiglitazone (1 % DMSO) and either 3 % ethanol or 3 % ethylene glycol. (**B**–**D**) Peak intensity profile of the rosiglitazone-bound PPARγ LBD compared to rosiglitazone-bound PPARγ LBD after addition of (**B**) GW9662, (**C**) ethanol, or (**D**) ethylene glycol. (**E**) Percentages of the number of visible and assignable peaks depending on bound chemical. (**F**) Crystal structure of PPARγ LBD bound to rosiglitazone in cartoon representation (PDB: 1FM6). Rosiglitazone is shown as spheres in the binding pocket, and residues only visible in the active state are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

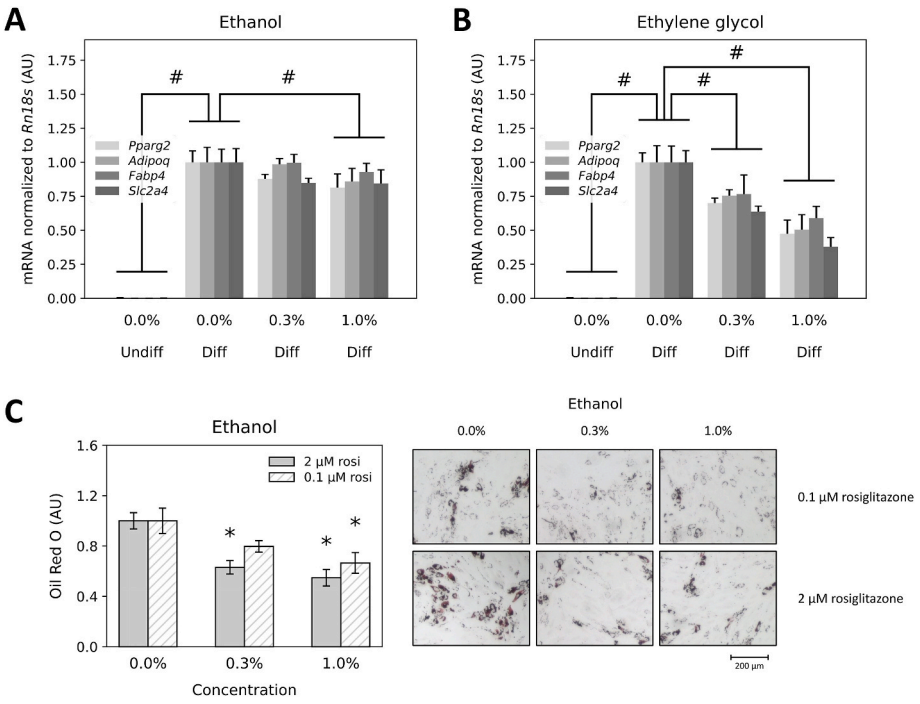

Fig. 2. Lipid accumulation and adipogenic marker mRNA in response to chemical exposure during adipocyte differentiation. C3H10T1/2 cells were differentiated for 6 days in the presence of 0, 0.3, or 1 % (**A**) ethanol or (**B**) ethylene glycol. Cells were compared to an undifferentiated control. Gene expression analysis by RT-qPCR was performed for adipocyte markers (*Pparg2*, *Adipoq*, *Fabp4*, *Slc2a4*) using SYBR Green assay ($n = 4$). (**C**) Differentiation of human ASCs was induced with either high (2 µM) or low (0.1 µM) concentrations of rosiglitazone in the adipogenic medium. During differentiation, cells were exposed to 0, 0.3, or 1 % ethanol. Lipids were stained with Oil Red O at day 12 of differentiation, visualized by microscopy, and quantified ($n = 4$). The graphs show means ± SEM. Asterisk (*) and hash (#) indicate statistically significant differences compared to the differentiated unexposed control cells using Dunnett's test or two-way ANOVA followed by Dunnett's test, respectively ($p < 0.05$).

of 0.3 or 1 % ethanol, using 0.1 µM or 2 µM rosiglitazone in the adipogenic medium (Fig. 2C). Lipid staining by Oil Red O showed a decrease in lipid accumulation in response to ethanol at the two rosiglitazone concentrations (two-way ANOVA, $p < 0.05$). Furthermore, the decrease was independent of rosiglitazone concentration as there was no statistically significant difference between the two rosiglitazone concentrations used (two-way ANOVA, $p > 0.05$).

3.3. Short-term regulation of aromatase in vitro by ethanol and ethylene glycol

To determine a potential acute effect of exposure to ethanol or ethylene glycol on aromatase expression, human A41 pre-adipocytes or adipocytes were exposed to concentrations of 1 % for 24 h (Fig. 3). In pre-adipocytes, ethanol had no effect, while ethylene glycol decreased aromatase mRNA levels (Fig. 3A). In mature adipocytes, ethanol exposure increased aromatase expression, and ethylene glycol exposure again resulted in a downregulation of aromatase mRNA levels (Fig. 3B). Exposure to either ethanol or ethylene glycol lowered mRNA levels of the two PPARγ target genes, *ADIPOQ* and *FASN*, which were used as indirect measures of PPARγ activity. Repeating the chemical exposure of A41 adipocytes in serum-free conditions produced a similar effect on aromatase expression as in conditions with serum (Supplemental Fig. 2).

3.4. Regulation of aromatase by ethanol and ethylene glycol in rats

The acute effects of ethanol and ethylene glycol were further explored *in vivo* by a two-day oral exposure in female rats, using the PPARγ antagonist GW9662 as a positive control for PPARγ-mediated effects. Ethanol and ethylene glycol were dosed in the drinking water at 10 % (v/v) and 0.75 % (v/v), respectively, and GW9662 (2 mg/day) was administered through a vehicle consisting of hazelnut chocolate cream

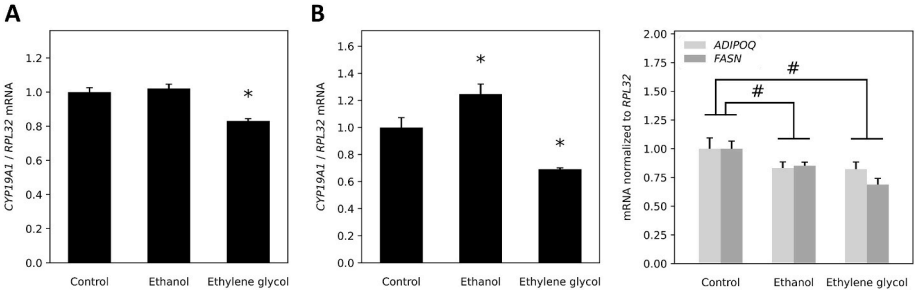

Fig. 3. Aromatase mRNA level in response to ethanol or ethylene glycol exposure. Gene expression analysis of aromatase (*CYP19A1*) and adipocyte markers (*ADIPOQ* and *FASN*) was performed by RT-qPCR using SYBR Green assay. (**A**) Undifferentiated or (**B**) differentiated A41 cells were exposed to 1 % ethanol or ethylene glycol for 24 h (both panels). The graphs present means ± SEM ($n = 3$). Asterisk (*) and hash (#) indicate statistically significant differences compared to control cells using Dunnett's test or two-way ANOVA followed by Dunnett's test, respectively ($p < 0.05$).

on biscuit. The vehicle was delivered to all four groups. There were no significant differences in fluid intake and weight gain between groups (Supplemental Fig. S3).

Expression levels of PPARγ (*Pparg*), fatty acid binding protein 4 (*Fabp4*), adiponectin (*Adipoq*), leptin (*Lep*), and interleukin 6 (*Il6*) were measured in subcutaneous mammary white adipose tissue (sWAT; Fig. 4A), visceral white adipose tissue (vWAT; Fig. 4B), and ovaries (Fig. 4C) by RT-qPCR. Aromatase (*Cyp19a1*) mRNA was also measured in adipose tissues (Fig. 4D) and ovaries (Fig. 4E). Chemical exposures had no effect on the expression of these genes, apart from an increase in leptin expression in sWAT in response to ethylene glycol exposure. Aromatase was very weakly expressed in rat adipose tissue, indicated by a lack of amplification in a substantial number of the technical replicates. However, specific *Cyp19a1* amplification in sWAT samples was confirmed in the TaqMan assay by agarose gel electrophoresis of the PCR product (Supplemental Fig. S4). In contrast, amplification of *Cyp19a1* using SYBR Green produced both *Cyp19a1* and non-specific amplicons.

4. Discussion

Genetic epidemiological studies of alcohol-induced breast cancer have shown interaction between the functional polymorphism *PPARG2* Pro12Ala and alcohol intake in relation to breast cancer in postmenopausal women in the prospective Diet, Cancer and Health cohort [9,11]. In these studies, postmenopausal women were at 13 % increased risk of breast cancer per 10 g alcohol per day if they were homozygous carriers of the wildtype *PPARG2* 12Pro allele, whereas variant *PPARG2* 12Ala carriers were not at increased risk of alcohol-related breast cancer [9]. The 12Ala variant is specific to the PPARγ2 isoform and encodes a PPARγ2 protein with altered ligand interaction [9,30]. Furthermore, serum levels of estrone sulphate (but not estrone) were positively correlated with alcohol intake among homozygous wildtype *PPARG2* 12Pro carriers, but not among variant allele carriers [9]. The observed interaction between *PPARG2* Pro12Ala and alcohol intake in relation to breast cancer places PPARγ2 and alcohol in the same biological mechanism of action of alcohol-related breast cancer. PPARγ2 is the main PPARγ isoform in adipose tissue and is a negative regulator of aromatase activity [19,21]. After menopause, adipose tissue is an important contributor to blood levels of sex hormones [31]. Alcohol dehydrogenase oxidizes ethanol to acetaldehyde and, in the Diet, Cancer and Health study, genetically determined slow oxidation of ethanol to acetaldehyde was associated with increased risk of alcohol-related breast cancer [7], suggesting that ethanol rather than acetaldehyde is the breast carcinogen. Based on this data, we hypothesized that alcohol causes breast cancer after menopause through a mechanism that modifies PPARγ2-dependent aromatase activity [9]. We furthermore hypothesized that other chemicals acting as PPARγ antagonists may have similar effects. Ethylene glycol was subsequently identified as a PPARγ antagonist *in vitro* [10]. The purpose of the present study was to assess the ability of ethanol and ethylene glycol to promote estrogen biosynthesis by affecting aromatase expression in adipose cells *in vitro* and *in vivo*.

We have shown by NMR that ethanol and ethylene glycol can have a small global effect on the active state of PPARγ, leading to chemical shifts and intensity changes without dislodging rosiglitazone from the LBD. Further, both chemicals display negative effects on adipogenesis. Short-term stimulation of human adipocytes with ethanol increased aromatase expression, however this was not the case for ethylene glycol. Female rats exposed orally to ethanol or ethylene glycol for two days did not exhibit altered gene expression of aromatase or PPARγ target genes in adipose tissue.

4.1. Interaction of ethanol and ethylene glycol with PPARγ

Activating ligands, such as rosiglitazone, stabilize the PPARγ LBD in the active state, leading to more visible peaks in 2D NMR spectra [21,32]. The same was observed in control experiments [21], where especially helix 12 became visible in the rosiglitazone-bound state, which has been described to be solvent exposed in the active state and buried in the ligand binding pocket in the repressive state [33]. Along with helix 12, peaks belonging to residues outlining the ligand binding pocket (helix 3, helices 5 to 7, C-terminal half of helix 10/11) also appeared [21]. These peaks were lost when PPARγ LBD was forced into the repressive state by the addition of repressors such as GW9662 or DEHPA

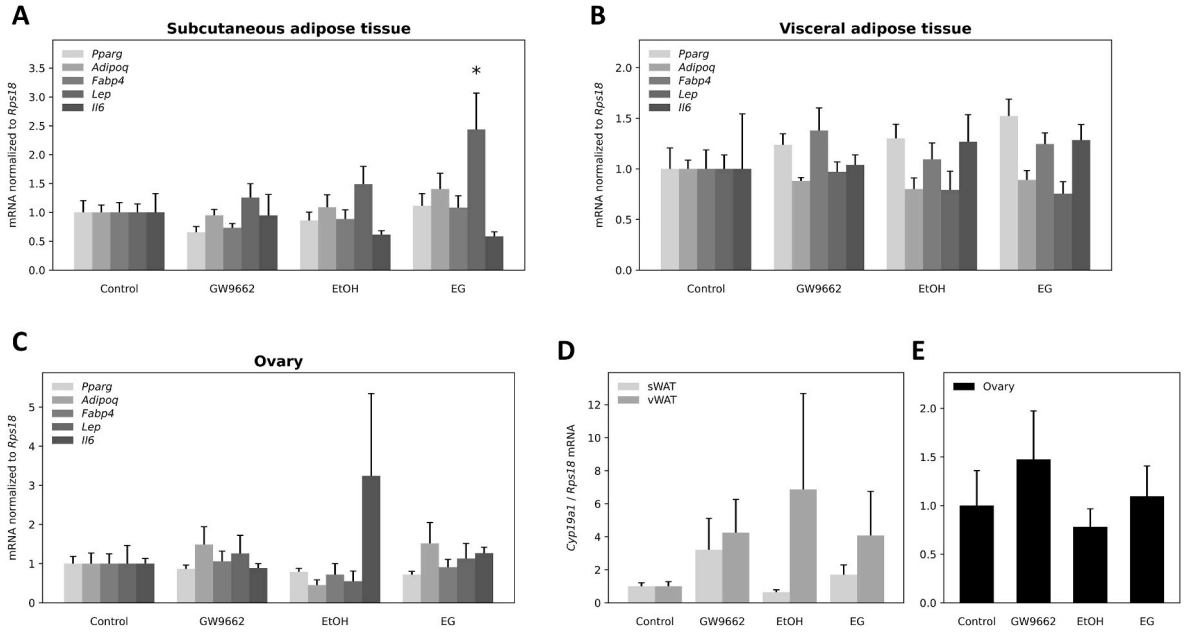

Fig. 4. Gene expression in rat tissues in response to short-term oral chemical exposure. Wistar rats were exposed to GW9662, ethanol, or ethylene glycol for 2 days before euthanasia. Gene expression analysis of *Cyp19a1*, *Pparg*, *Fabp4*, *Adipoq*, *Lep*, and *Il6* in (**A**,**D**) mammary adipose tissue, (**B**,**D**) visceral adipose tissue, and (**C**,**E**) ovaries was performed by RT-qPCR using TaqMan assay. The graphs present means ± SEM ($n = 6$; $n = 4$–6 for *Cyp19a1* in adipose tissue). Asterisk (*) indicates statistically significant difference compared to control rats using Dunnett's test ($p < 0.05$).

[21]. In contrast, addition of ethanol or ethylene glycol to PPARγ LBD did not seem to fully destabilize the rosiglitazone-bound active state. Peaks from helix 12 and the residues of the ligand binding pocket remained visible. In the case of ethanol, and to a lesser extent ethylene glycol, these peaks lost intensity, maybe suggesting repression. It has been suggested that significant changes in the function of a protein may occur in response to ethanol binding, if binding occurs in the regions of a protein that are involved in binding of other molecules [34]. We observe more pronounced chemical shift changes in the presence of rosiglitazone than in its absence. In the presence of rosiglitazone not all peaks shift in the same direction in the same pattern, speaking against a mere overall solution effect. It may therefore be that rosiglitazone, ethanol, and ethylene glycol affect each other in their interaction with the PPARγ LBD, without leading to a dissociation of rosiglitazone from the binding pocket. The data do not give obvious indications of a repression of PPARγ as observed for other known repressors. Repression may therefore be less strong or occur through an indirect or LBD-independent mechanism. Furthermore, it should be noted that metabolites of ethanol and ethylene glycol may also affect PPARγ function.

4.2. Effect of ethanol and ethylene glycol on adipogenesis

We recently provided evidence for a link between PPARγ antagonist-induced loss of adipogenic capacity and elevated aromatase expression in human ASCs [21], and we therefore studied the effects of ethanol and ethylene glycol on adipogenesis. The impaired adipogenesis in response to ethanol and ethylene glycol supports the reported inhibitory effect of these chemicals on PPARγ [9,10] and implies that a stimulation of aromatase expression may result from this. Ethanol has been demonstrated to inhibit adipogenesis in human ASCs at a concentration of 50 mM, corresponding to 0.3 % (v/v), which is consistent with the present results [35]. In contrast, 100 mM ethanol has been shown to induce adipocyte differentiation of the mouse OP9 cell line [36]. These inconsistencies might result from differential expression and activity of ethanol-metabolizing enzymes, which have been shown to be expressed in adipocytes [35].

Ethanol has numerous biological effects and is believed to act on many different proteins making it difficult to identify its direct targets [37]. For example, ethanol may affect adipogenesis through its inhibitory effect on insulin action [38]. The inhibition of adipogenesis by ethanol in primary human ASCs occurred independently of the rosiglitazone concentration, which is in contrast to the effects of other studied PPARγ inhibitors, such as GW9662, Cosan 528, and DEHPA [21]. This indicates that PPARγ-independent mechanisms of ethanol may contribute to the impaired adipogenesis.

4.3. Short-term regulation of aromatase by ethanol and ethylene glycol in vitro

An acute effect of PPARγ antagonists on aromatase expression has previously been reported [21]. Whether a similar effect would occur in response to ethanol or ethylene glycol was therefore investigated. Like other PPARγ antagonists, ethanol increased aromatase mRNA in *PPARG*-expressing A41 adipocytes, but not in A41 pre-adipocytes, suggesting that PPARγ may be involved. Consistent with this, a study has demonstrated that ethanol exposure increases aromatase expression in the MCF-7 human breast cancer cell line [39]. Surprisingly, ethylene glycol reduced aromatase mRNA in the A41 adipocytes, despite inhibiting PPARγ activity. The corresponding decrease in aromatase mRNA at the pre-adipocyte stage, where *PPARG* is lowly expressed, suggests that the ethylene glycol-stimulated effects on aromatase were PPARγ-independent.

While we were limited to study the regulation of aromatase at mRNA, but not protein, levels in our study, it has previously been demonstrated that there is a strong consistency between aromatase mRNA, protein, and activity levels as well as estrogen production in pre-adipocytes [18,40,41], suggesting that the aromatase mRNA levels are a good predictor of estrogen synthesis.

4.4. In vivo regulation of aromatase by ethanol and ethylene glycol

Acute exposure of rats to GW9662, ethanol, or ethylene glycol did not result in any apparent effects on mRNA levels of aromatase or aromatase-associated cytokines [42], nor on *Pparg* or PPARγ target genes. Aromatase expression was expected to increase in response to PPARγ antagonist exposure as previously demonstrated in cultured adipocytes [21]. Other *in vivo* studies have reported that ethanol consumption downregulates *Pparg*, *Fabp4*, and *Adipoq* and upregulates *Cyp19a1* and *Il6* in rodent adipose tissue [14,15,23,36,43–45], while ethylene glycol downregulates renal *Pparg* and increases serum IL-6 [12]. Ethanol-induced upregulation of aromatase was demonstrated at the protein level and occurred after 8 weeks of *ad libitum* access to 13 % ethanol in the form of red wine or ethanol [23]. Because exposure was chronic, indirect effects, such as impaired adipogenesis, possibly contributed to the upregulation of aromatase.

The lack of effects in the current study may be explained by the short duration of exposure. In most rodent studies on these chemicals, the animals were exposed for several weeks [12–15,23]. However, acute exposures have been shown to affect gene expression. For instance, intraperitoneal administration of 2 mg/kg GW9662 for 26 h or 3.5 g/kg ethanol for 3 h increased *Il6* expression in rodent hippocampus [46,47]. In addition, oral gavage with 7 g/kg bw ethanol altered levels of hepatic triglycerides and cholesterol in mice as well as the number of CD68 positive macrophages in liver after 6 h [48].

Female rats were selected in the present study because breast cancer is most common in females. Since the phase of estrous cycle for each animal was not determined, it is unclear whether estrous cycle influenced the activity or expression of PPARγ and aromatase. Fluctuations in adipose tissue *Pparg* expression over the course of estrous cycle have been found in rodents [49,50]. Therefore, the estrous cycle may mask potential chemical-induced effects on expression of *Pparg* and its target genes, and possibly also *Cyp19a1*. However, it has been reported that long-term oral GW9662 exposure of sexually mature female mice decreased mRNA expression of *Pparg*, *Adipoq*, and *Fabp4* more than ten-fold in mammary adenocarcinomas [24]. In addition, aromatase mRNA has been shown to increase significantly in the adipose tissue from female mice placed on a high-fat diet for 16 weeks compared to mice on a low-fat diet [51], suggesting minimal interference of the estrous cycle on adipose tissue aromatase expression.

Aromatase mRNA levels were low in rat adipose tissue, which has also been observed in mouse adipose tissue cells [21] and tissue [52], resulting in large variation within each group. A transgenic humanized aromatase mouse model has been generated to mimic human tissue-specific patterns of aromatase expression and estrogen production, and this would be an ideal model for studying effects on aromatase mRNA in adipose tissue [52].

5. Conclusion

Our results suggest that ethanol and ethylene glycol can inhibit PPARγ activity, potentially through a direct interaction with PPARγ or through weak interference with rosiglitazone binding. Thus, exposure to ethanol or ethylene glycol decreased expression of *Pparg2* and PPARγ2 target genes in a mouse mesenchymal stem cell line. Ethanol exposure also inhibited lipid accumulation during differentiation in primary human ASCs. Short-term ethanol exposure increased aromatase expression in human adipocytes, whereas ethylene glycol exposure decreased aromatase expression, likely through a PPARγ-independent mechanism. There were no acute effects of GW9662, ethanol, or ethylene glycol on aromatase expression in rat adipose tissue or ovary.

CRediT authorship contribution statement

Jacob Ardenkjær-Skinnerup: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Daniel Saar:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Sofie Christiansen:** Writing – review & editing, Resources, Methodology. **Terje Svingen:** Writing – review & editing, Resources. **Niels Hadrup:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Kristy A. Brown:** Writing – review & editing, Supervision, Resources, Methodology. **Brice Emanuelli:** Writing – review & editing, Supervision, Resources, Methodology. **Birthe B. Kragelund:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis. **Gitte Ravn-Haren:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Ulla Vogel:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101742.

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