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# Metformin decreases hyaluronan synthesis by vascular smooth muscle cells

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Accepted 2 October 2019  
Published Online First  
30 October 2019

## ABSTRACT

Metformin is the first-line drug in the treatment of type 2 diabetes worldwide based on its effectiveness and cardiovascular safety. Currently metformin is increasingly used during pregnancy in women with gestational diabetes mellitus, even if the long-term effects of metformin on offspring are not exactly known. We have previously shown that high glucose concentration increases hyaluronan (HA) production of cultured human vascular smooth muscle cells (VSMC) via stimulating the expression of hyaluronan synthase 2 (*HAS2*). This offers a potential mechanism whereby hyperglycemia leads to vascular macroangiopathy. In this study, we examined whether gestational metformin use affects HA content in the aortic wall of mouse offspring *in vivo*. We also examined the effect of metformin on HA synthesis by cultured human VSMCs *in vitro*. We found that gestational metformin use significantly decreased HA content in the intima-media of mouse offspring aortas. In accordance with this, the synthesis of HA by VSMCs was also significantly decreased in response to treatment with metformin. This decrease in HA synthesis was shown to be due to the reduction of both the expression of *HAS2* and the amount of HAS substrates, particularly UDP-*N*-acetylglucosamine. As shown here, gestational metformin use is capable to program reduced HA content in the vascular wall of the offspring strongly supporting the idea, that metformin possesses long-term vasculoprotective effects.

## INTRODUCTION

Metformin (*N,N*-dimethylbiguanide) is endorsed as the first-line drug in the treatment of type 2 diabetes worldwide.<sup>1</sup> During pregnancy of patients with type 2 diabetes, metformin treatment is usually replaced with insulin due to the extensive clinical experience on the safety of insulin.<sup>2</sup> However, today metformin is increasingly used also during pregnancy in women with gestational diabetes mellitus,<sup>3</sup> even though its long-term effects on the offspring have raised some concern.<sup>2</sup> For example, the effect of gestational metformin use on the vascular system of the offspring is not completely known.

The glucose-lowering effect of metformin is mainly caused by its capability to inhibit hepatic

## Significance of this study

### What is already known about this subject?

- ▶ Metformin is the first-line drug in the treatment of type 2 diabetes.
- ▶ Long-term effects of its use in gestational diabetes mellitus are not exactly known.
- ▶ High glucose concentration increases the production of hyaluronan by vascular smooth muscle cells.
- ▶ Accumulated hyaluronan contributes to the development of microvascular and macrovascular complications of patients with diabetes.

### What are the new findings?

- ▶ Gestational metformin use significantly decreases intima-media hyaluronan content in mouse offspring aortas.
- ▶ The gestational use of metformin is able to program reduced hyaluronan content in the vascular wall of offspring.
- ▶ Metformin decreases the synthesis of hyaluronan by human vascular smooth muscle cells.
- ▶ Decreased production of hyaluronan after metformin use is due to reduction of hyaluronan synthase 2 and its substrates.

### How might these results change the focus of research or clinical practice?

- ▶ Gestational use of metformin may possess long-term vasculoprotective effects.

gluconeogenesis and to improve glucose uptake in peripheral tissues. The effect of metformin on blood glucose has been shown to be mediated via different pathways, mainly via the 5'-AMP-activated protein kinase (AMPK) pathway.<sup>4</sup> Metformin has also been shown to decrease the risk of cardiovascular events such as myocardial infarction<sup>5</sup> and stroke<sup>6</sup> of patients with diabetes independent of its glucose-lowering effects. Regarding the cardiovascular system, metformin has been shown to exhibit various beneficial actions such as reduction of vascular inflammation and inhibition of adverse remodelling of the myocardium.<sup>7</sup> However,



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**To cite:** Sainio A, Takabe P, Oikari S, et al. *J Investig Med* 2020;**68**:383–391.

the precise mechanisms behind these so-called pleiotropic effects of metformin are still poorly understood.<sup>8</sup>

We have demonstrated yet that the sera of both patients with type 1<sup>9</sup> and type 2<sup>10</sup> diabetes stimulate the synthesis of hyaluronan (HA) in cultured human aortic smooth muscle cells (SMC). More recently we showed that high glucose concentration modulates the reorganization of the extracellular matrix (ECM) by vascular smooth muscle cells (VSMC) via stimulating HA synthesis.<sup>11</sup>

HA is an unbranched glycosaminoglycan, whose structure comprised repeating polysaccharides, namely D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc). In mammals, HA is synthesized in the inner face of plasma membrane by 3 hyaluronan synthases (HAS1–3).<sup>12</sup> As substrates, HAs use the cytosolic pool of UDP-sugars including UDP-glucuronic acid (UDP-GlcUA) and UDP-N-acetylglucosamine (UDP-GlcNAc).<sup>13</sup> It has been reported that depletion of either of these substrates can become a limiting factor in HA synthesis.<sup>14</sup> In inflammatory vascular diseases such as atherosclerosis, both the synthesis and catabolism of HA are increased.<sup>15</sup> Furthermore, in the vasculature of patients with diabetes accumulated HA contributes to the development of microvascular and macrovascular complications.<sup>16</sup> There is also evidence that increased HA content in the vascular wall has a role in aneurysms.<sup>17</sup>

In this study, we found that gestational metformin use significantly decreases HA content in the intima-media of aorta of mouse offspring in vivo. We also showed that metformin reduces the production of HA, the expression of *HAS2*, and the cellular pool of UDP-sugars in cultured human VSMCs in vitro.

## MATERIALS AND METHODS

### Tissue samples

The aorta samples were obtained from previous experiments conducted by Salomäki *et al*,<sup>18</sup> where C57/BL6NHsd mice were prenatally exposed to metformin. Dose of 300 mg/kg of metformin (Sigma-Aldrich, St Louis, Missouri, USA) or vehicle (tap water) was administered orally to the dams from embryonic day 0.5 (E0.5) to E17.5. The concentration of metformin in the serum of fetuses at E18.5 was 130+21 nmol/L and in dams 174+114 nmol/L, respectively.<sup>19</sup> In the end of the experiment, euthanasia of the offspring was induced with a premade mixture of medetomidine:ketamine:saline in ratio of 2:3:5. All animal work was planned and performed according to the Finnish Act on the Use of Animals for Experimental Purposes. The aortas of metformin exposed offspring (n=3) and their unexposed control offspring (n=3) were all from male offspring. The aortas were snap-frozen in liquid nitrogen, embedded in paraffin and cut into 5 µm sections, thereafter they were used for H&E and HA staining.

### HA staining

The aorta sections were stained with biotinylated HA-binding complex of the cartilage aggrecan G1 domain and link protein (bHABC), a probe specific for HA, and the staining was performed using a standard protocol.<sup>20</sup> Briefly, the sections were incubated in bHABC (2.5 µg/mL in 1% bovine serum albumin) overnight at 4°C. The probe was prepared in the laboratory of Institute of Biomedicine of

Kuopio, University of Eastern Finland, Kuopio, Finland, as previously described.<sup>21</sup> Thereafter, the slides were washed with phosphate buffered saline (PBS) and treated with avidin-biotin-peroxidase (Vector Laboratories, Irvine, CA) (1:200) for 1 hour at room temperature. After washes with PBS, the color was developed with 3,3'-diaminobenzidine (Sigma Chemical, St Louis, MO) for 5 minutes at room temperature. The slides were counterstained with Mayer's hematoxylin for 2 minutes. HA staining was quantified with reciprocal intensity using ImageJ software.<sup>22 23</sup>

### Culture of human VSMCs

Three established cell lines, originally derived from human VSMCs of umbilical cords of healthy newborn babies, were a generous gift from Dr Saara Ihalainen (Protein Chemistry Unit, Institute of Biomedicine/Anatomy, University of Helsinki, Helsinki, Finland).<sup>24</sup> The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland) containing 10% (v/v) fetal bovine serum (Biochrom, Berlin, Germany), 100 IU/mL penicillin and 100 µg/mL streptomycin (Sigma, Saint Louis, Missouri, USA), and 2 mM L-glutamine (Sigma, Steinheim, Germany). The cells were grown in hyperglycaemic conditions with D-glucose concentration of 25 mmol/L, and used between passages 17 and 22. All the 3 VSMC lines were used in cell migration assay, effect of metformin on the proliferation of human VSMCs in culture, HA assay, RNA extraction and reverse transcription-quantitative PCR for *HAS1-3* (*HAS1-3* as italic), and high-performance liquid chromatography analysis of UDP-sugars.

### Cell migration assay

The scratch wound assay was used to examine the effect of metformin on human VSMC migration.<sup>19</sup> Briefly, on day 1, a total of 42,000 VSMCs were plated on 12-well plates (Starlab International, Hamburg, Germany) and placed for 48 hours in the incubator to reach the confluence. Thereafter, the medium was changed and metformin at concentrations of 1, 5 and 10 mmol/L was added to the cultures. No metformin was added in the fresh control culture medium. On the next day, the cell layer in each well was scraped with a 10 µL pipet tip in a straight line to create a 'scratch'. Cell debris was removed by washing the cultures once with 1 mL of the culture medium; thereafter, fresh culture medium with metformin at different concentrations (1, 5 and 10 mmol/L) was added. Images of the scratches were acquired at 0, 24, and 48 hours after the wounding using a Nikon Eclipse TS100 inverted microscope (4× objectives) and a Nikon DS-3 camera system. The relative area of gap closure at various time points was analysed using ImageJ software.<sup>23</sup>

### Effect of metformin on the proliferation of human VSMCs in culture

The effect of metformin on the proliferation of human VSMCs in culture was tested using 3 different concentrations (1, 5, and 10 mmol/L) of metformin. On day 1, a total of 300,000 cells were plated on 25 cm<sup>2</sup> tissue culture flask (Thermo Fisher Scientific, Rochester, NY), and the cells were left to settle for 24 hours. On the next day, the medium and metformin at different concentrations were changed to the cultures. No metformin was added to the fresh medium

of the control cultures. The cells were cultured for 48 hours, where after the cell number was counted using an automated cell counter (BioRad TC20, Hercules, CA, USA).

### HA assay

On day 1, the human VSMC lines were seeded on 25 cm<sup>2</sup> culture flasks (Thermo Fisher Scientific), 300,000 cells per flask. On the following day, fresh medium containing 1, 5, or 10 mmol/L of metformin was changed to the cultures. No metformin was added into the fresh control culture medium. After 48 hours' incubation, the media were collected and the amount of HA was determined using a sandwich-type enzyme-linked sorbent assay (ELSA) as previously described in detail.<sup>25</sup>

### RNA extraction and reverse transcription-quantitative PCR for *HAS1–3*

Expression of *HAS1–3* was measured from the same cultures that were used for HA assay (see above). Total RNA was extracted using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The amount of RNA was measured using a NanoDrop spectrophotometer (ThermoScientific, Waltham, MA). Quantitative PCR (qPCR) was performed as previously described with minor modifications.<sup>26</sup> Briefly, the synthesis of cDNA was performed with 1 µg of total RNA as a template. Messenger RNAs were analysed with Absolute MAX 2-step QRT-PCR SYBR Green Kit (ABgene, Epsom Surrey, UK), with specific primers shown in table 1 using MX3000P thermal cycler (Stratagene, La Jolla, CA). Fold inductions were calculated using the formula  $2^{-(\Delta\Delta Ct)}$  with acidic riboprotein PO (*Arpo*) as a control gene.

### High-performance liquid chromatography analysis of UDP-sugars

On day 1, a total of 300,000 human VSMCs were plated on 25 cm<sup>2</sup> culture flasks (Thermo Fisher Scientific). On the next day, the culture medium was changed into a new one which contained 1, 5, or 10 mmol/L of metformin. After 48 hours, the cells were analysed for UDP-sugars as previously described.<sup>27</sup> Briefly, the cells were placed on ice and washed with cold PBS, where after they were scraped into cold PBS, followed by sonication using SONO-PULS (Bandelin, Berlin, Germany). After sonication, small aliquots were collected and their total protein content was measured with Pierce BSA protein assay kit (Thermo Scientific). Total protein amounts were used to normalise

the UDP-sugar results. The rest of the solution was centrifuged (6000×g for 20 minutes), and further purified with Supelclean Envi-Carb SPE tubes (Sigma, Saint Louis, MO) as previously described.<sup>28</sup> Subsequently, the eluted samples were evaporated by vacuum centrifugation and dissolved in water for anion exchange high-performance liquid chromatography with a CarpoPac PA1 column (Dionex, Thermo Fisher Scientific) as previously described.<sup>27</sup>

### Statistical analysis

Unpaired Student's t-test was employed in statistical analyses. All p values <0.05 were considered statistically significant.

## RESULTS

### Intima-media thickness of offspring aorta is not affected by gestational metformin exposure

The aorta samples of the gestational metformin exposed mice offspring were stained with H&E and the intima-media thickness was determined. They were then compared with the aortas of non-treated control mice offspring. The results revealed no significant difference in the relative intima-media thickness between the aforementioned groups (figure 1A–C).

### Gestational metformin use decreases HA content in the aorta of mouse offspring

The aorta samples of mouse offspring were stained for HA, and its content in the aortas was determined using ImageJ software.<sup>22–23</sup> The intima-media of the control aortas showed moderate HA staining, while the intima-media of the aortas derived from the offspring exposed to gestational metformin was almost HA negative (figure 2A,B). The reduction in the HA content in the intima-media of aortas derived from metformin exposed offspring was statistically significant (p<0.01) (figure 2C).

### Migration and proliferation of human VSMCs in response to metformin

The results obtained from the migration assay showed that metformin affects the capability of cultured human VSMCs to migrate (figure 3A–H). The assay was quantified after 48 hours (figure 3I), and a statistically significant decrease in the migration of the cells exposed to 10 mmol/L of metformin was observed (figure 3H,I). In contrast, metformin had no statistically significant effect on the proliferation of the cells (figure 3J).

### Production of HA is reduced in response to metformin

To examine the possible effect of metformin on HA production by cultured human VSMCs, the amount of secreted HA was measured using ELSA (figure 4). The results revealed a statistically significant reduction in HA production by the cells exposed to metformin at the concentrations of 5 and 10 mmol/L. Also a tendency towards a lower HA production was observed already at the metformin concentration of 1 mmol/L (figure 4).

### Metformin decreases *HAS2* expression

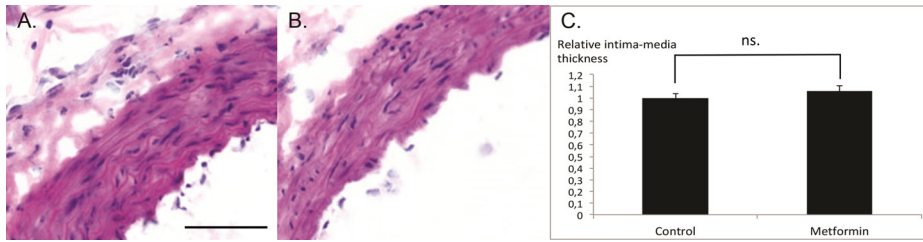
To verify whether the decrease in HA production by cultured human VSMCs in response to metformin could be due to

**Table 1** Primer sequences for *HAS1–3* RT-qPCR

Gene name	Primer sequence (5' to 3')
<i>HAS1</i>	Forward: CAAGATTCTTCAGTCTGGAC Reverse: TAAGAACGAGGAGAAAGCAG
<i>HAS2</i>	Forward: CAGAATCAAACAGACAGTTC Reverse: TAAGGTGTTGTGTGACTG
<i>HAS3</i>	Forward: CTTAAGGGTGTCTTGCTTGC Reverse: GTTCGTGGGAGATGAAGGAA
<i>Arpo</i>	Forward: AGATGCAGCAGATCCGCAT Reverse: GTGGTGATACCTAAGCCTG

*Arpo*, acidic riboprotein PO; *HAS*, hyaluronan synthase; RT-qPCR, reverse transcription-quantitative PCR.





**Figure 1** Effect of gestational metformin use on the intima-media thickness of the aorta of the offspring. (A) A representative image of H&E staining of aorta sample derived from a control mouse offspring not exposed to metformin. (B) A representative image of H&E staining of aorta sample derived from a gestational metformin-treated mouse offspring. Scale bar 50  $\mu$ m. (C) Quantification of relative intima-media thickness of aortas derived from a control mouse offspring (n=3) and from a gestational exposed offspring (n=3). The intima-media thickness of the aortas was measured from the endothelial surface to the adventitia in 5 different fields of samples of each animal. Note that there is no significant (ns) difference in the intima-media thickness of aortas between the 2 groups. The data represent mean $\pm$ SD.

the decrease in the mRNA expression of different *HASes*, reverse transcription-qPCR analyses for *HAS1-3* were performed (figure 5A–C). VSMCs were found to express all 3 *HAS* isoforms, but the expressions of *HAS2* and *HAS3* were most prominent. However, metformin decreased statistically significantly only the expression of *HAS2*.

#### Metformin decreases the cytosolic UDP-sugar content of VSMCs

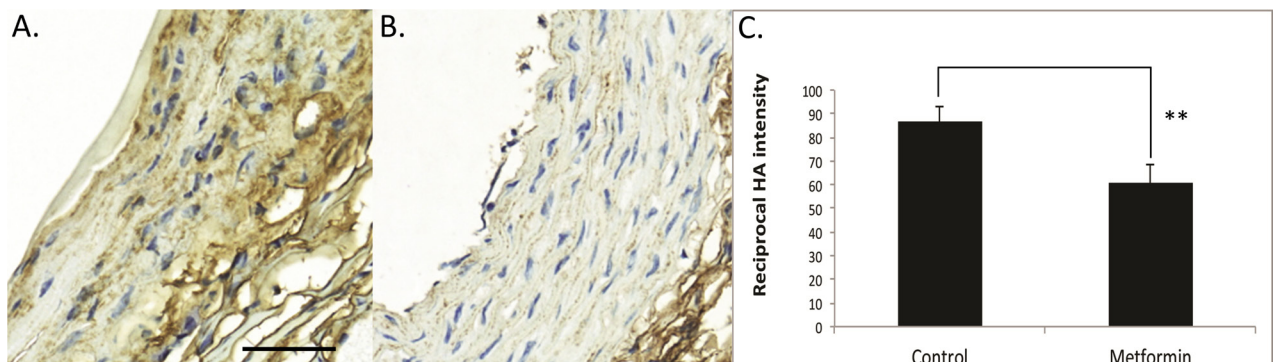
The production rate of HA can be dependent on the amount of HA synthesizing enzymes, *HASes*<sup>29</sup> and/or on the supply of their substrates (eg, GlcUA and GlcNac).<sup>14</sup> The results showed that the amounts of different UDP-sugars were markedly decreased in response to metformin (figure 6A–C). Note that the basal level of UDP-GlcNac was 3 times higher than that of UDP-GlcUA. After metformin exposure, particularly the level of UDP-GlcUA precursor, UDP-D-glucose (UDP-Glc), and the level of UDP-GlcNac were statistically significantly reduced (figure 6A,C). Furthermore, a clear trend of dose-dependent reduction in the amount of UDP-GlcUA in VSMCs was evident in response to metformin exposure.

#### DISCUSSIONDISCUSSION

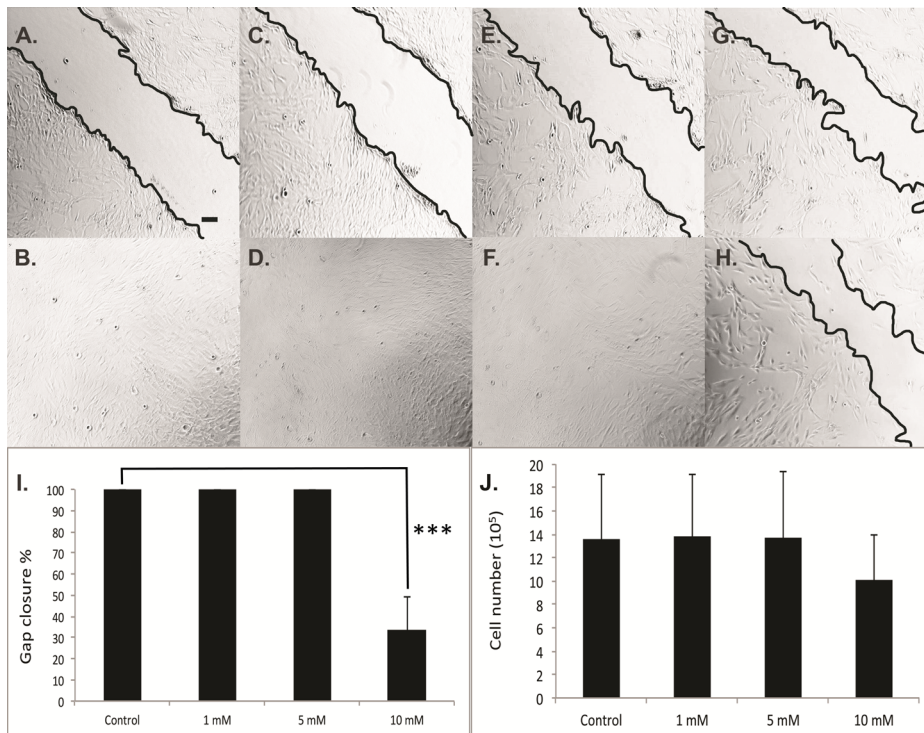
It has previously been shown that metformin can cross human placenta and that the concentration of metformin

in fetal cord blood and in the maternal plasma is nearly the same.<sup>30</sup> As the use of metformin before and during the pregnancy has been increasing, its safety and long-term effects on the offspring have been of great interest.<sup>2</sup> This is due to the vast variety of possible actions of metformin targeting different cells and tissues.<sup>31</sup> Regarding the effect of metformin on the developing vascular system of human fetuses, only limited data are available. Recently, it was demonstrated that metformin has no intrinsic effects on the vasculature of rat offspring.<sup>32</sup> However, in this study we have shown that gestational metformin use causes a marked decrease in HA content in the aorta of mice offspring.

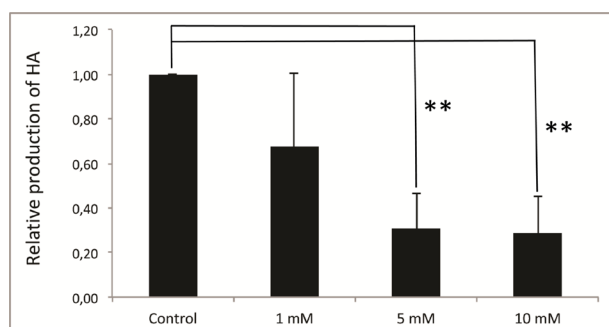
In normal blood vessels, HA is present in low quantities, but its production increases dramatically in different vascular diseases, including diabetes-associated vascular macroangiopathy.<sup>33,34</sup> We have previously shown that the sera of both patients with type 1<sup>9</sup> and type 2<sup>10</sup> diabetes stimulate the synthesis of HA by cultured human aortic SMCs. We have also previously demonstrated that high glucose concentration (mimicking diabetes) stimulates HA production and interferes with the remodelling of the vascular ECM by cultured VSMCs.<sup>11</sup> In addition, induction of VSMC proliferation and migration has been shown to be modulated as a consequence of HA accumulation.<sup>35</sup> In atherosclerosis, the increased amount of HA together with the upregulated expression of *HASes* and HA receptors has



**Figure 2** HA content in the mouse aorta of the offspring whose mothers were treated with metformin during pregnancy. Representative images of HA staining of aorta samples derived from a control mouse offspring (A) and from a gestational metformin exposed mouse offspring (B). Scale bar 50  $\mu$ m. (C) Quantification of the HA content in the aortas derived from control (n=3) and metformin exposed (n=3) mouse offspring. The images were analysed using ImageJ software.<sup>22,23</sup> Note that gestational metformin treatment statistically significantly (\*\*p<0.01) reduces the HA content in the aorta of mouse offspring. The data represent mean $\pm$ SD. HA, hyaluronan.



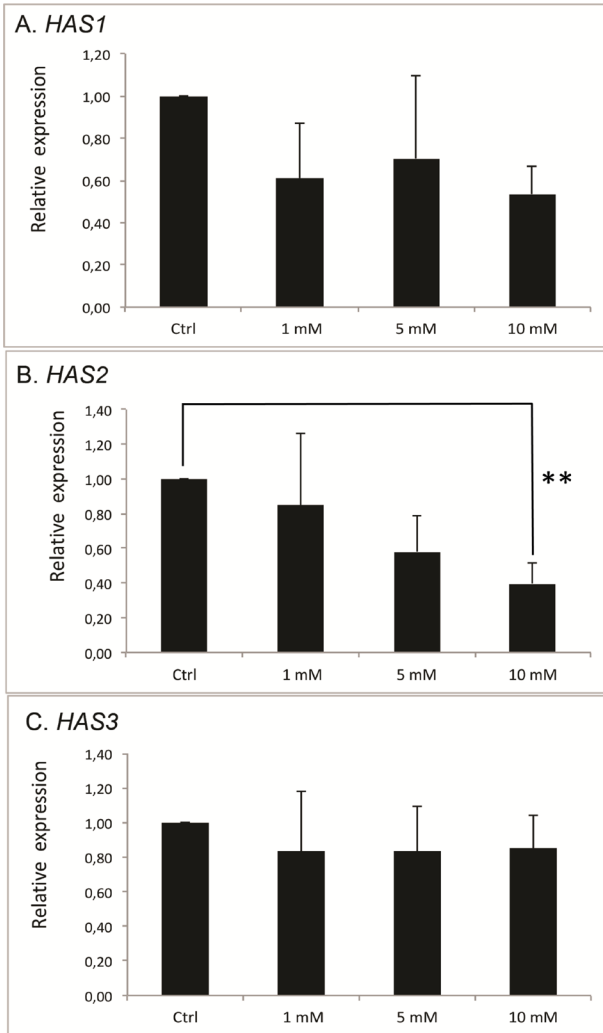
**Figure 3** Metformin treatment decreases the migration of cultured human vascular smooth muscle cells (VSMC), but has no significant effect on their proliferation. Scratch wound assay was used to measure the effect of metformin on the capability of cultured human VSMCs to migrate.<sup>19</sup> Representative images at the time points 0 and 48 h are shown. (A, B) Representative VSMC cultures at the time points 0 and 48 h not exposed to metformin. (C, D) Representative VSMC cultures at the above time points exposed to metformin at the concentration of 1 mmol/L. (E, F) Representative VSMC cultures at the above time points exposed to metformin at the concentration of 5 mmol/L. (G, H) Representative VSMC cultures at the above time points exposed to metformin at the concentration of 10 mmol/L. Scale bar 100  $\mu$ m. (I) Quantification of the gap closure at the 48 h time point. The experiments were conducted with 3 VSMC lines and as 3 replicate assays. The results are shown as per cent in comparison to gap size in the beginning of the experiment. Note that the gap closure is complete at the 48 h time point in all cultures except in the cultures exposed to 10 mmol/L of metformin (\*\*\*) ( $p < 0.001$ ). (J) The number of VSMCs of control and metformin exposed (1, 5 and 10 mmol/L) cultures at the 48 h time point ( $n=3$ ). Note that there is no statistically significant difference in the cell number between any of the cultures. The data represent mean  $\pm$  SD.



**Figure 4** Metformin decreases HA production by cultured human vascular smooth muscle cells (VSMC). The relative production of HA by human VSMC control and metformin exposed (1, 5 and 10 mmol/L) cultures during 48 h as measured with enzyme-linked sorbent assay (ELSA), a sandwich-type enzyme-linked sorbent assay. The experiments were conducted with 3 VSMC lines and as 3 replicate assays. Note that there is a statistically significant reduction in HA production by cultured human VSMCs exposed to metformin at the concentrations of 5 and 10 mmol/L (\*\* $p < 0.01$ ). The data represent mean  $\pm$  SD. HA, hyaluronan.

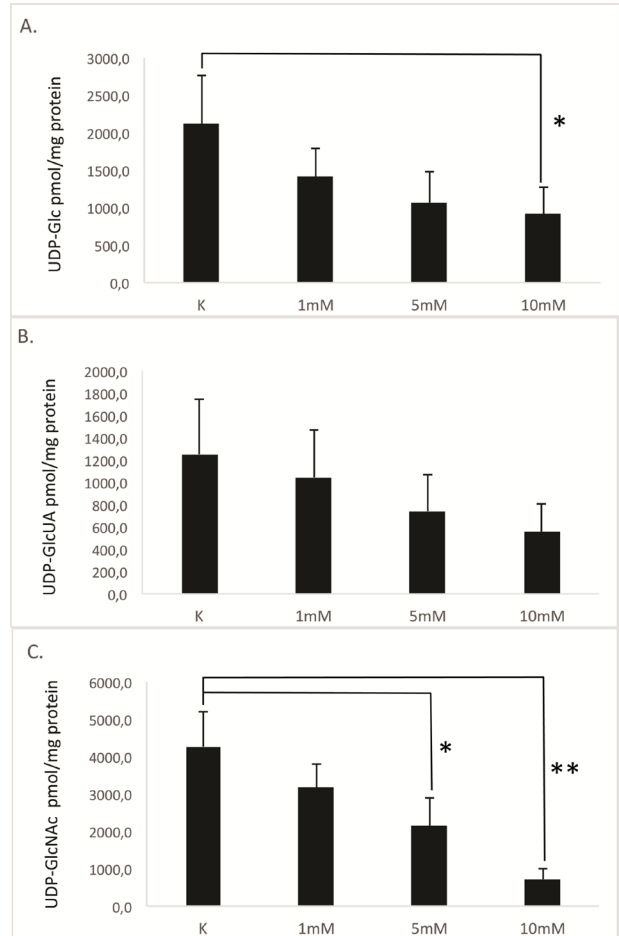
been shown to result in disruption of vascular integrity.<sup>36 37</sup> Particularly, overexpression of *HAS2* has been shown to be associated with progression of atherosclerosis<sup>38</sup> and neointimal formation after vascular injury.<sup>39</sup> There is also evidence that increase in vascular HA content contributes to the development of aneurysms<sup>17</sup> via influencing the biomechanical resistance of the vascular wall.<sup>40</sup> Accumulation of HA is also known to possess both local and systemic proinflammatory actions such as increased retention of macrophages and promotion of Th1 polarization, respectively.<sup>15</sup> As a summary, the result of this study that gestational metformin exposure of mouse offspring is able to program the HA content in the aorta of the offspring can be regarded as a desirable outcome.

The synthesis and catabolism of HA in cells are dynamic processes, and they are the prerequisites for normal cellular functions.<sup>41</sup> However, dysregulated HA metabolism can be regarded as a proatherosclerotic factor.<sup>15</sup> Various growth factors and cytokines such as platelet-derived growth factor,<sup>42</sup> transforming growth factor-beta (TGF- $\beta$ ),<sup>43</sup> and interleukin 1 $\beta$ <sup>44</sup> have been shown to be able to increase *HAS* expression, and subsequently HA production and accumulation in vascular wall. Regarding metformin, it has been reported to reduce HA synthesis



**Figure 5** Expression of *HAS2* is significantly decreased by cultured human vascular smooth muscle cells (VSMC) in response to metformin. In order to evaluate whether the observed reduction in hyaluronan (HA) production by cultured human VSMCs was dependent on the amount of HASEs, the relative expression of *HAS1–3* was examined in metformin exposed cultures at the 48 h time point (A–C). The experiments were conducted with 3 VSMC lines and as 3 replicate assays. As seen in the image (B), a significant decrease at the mRNA level of *HAS2* was detected in cultured human VSMC cultures exposed to metformin at the concentration of 10 mmol/L (\*\* $p < 0.01$ ).

in VSMCs via downregulating the activity of the main HA synthesizing enzyme, namely *HAS2*.<sup>45</sup> In our present study, we did not examine the activation level of *HAS2*, but we noticed a marked decrease in its expression. Interestingly, no similar reduction was found in *HAS1* or *HAS3* expressions. It has previously been shown that *HAS2*, but not *HAS1* and *HAS3*, is phosphorylated by AMPK.<sup>45</sup> The specificity of this interaction was further demonstrated to be based on AMPK phosphorylation consensus motif present only in *HAS2* protein, namely at Thr-110.<sup>45</sup> However, no alterations in the expression of genes involved in HA metabolism, including *HAS1–3*, have been reported after metformin treatment.<sup>45</sup> We also observed a significantly



**Figure 6** Metformin exposure of human vascular smooth muscle cells (VSMC) markedly reduces the amount of cytosolic UDP-sugars, particularly UDP-Glc and UDP-GlcNAc in the cells. The quantity of the UDP-sugar pool was examined in human VSMC cultures exposed to metformin (A–C). The experiments were conducted with 3 VSMC lines and as 3 replicate assays. The levels of UDP-glucuronic acid (UDP-GlcUA) precursor (B), UDP-D-glucose (UDP-Glc) (A) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) (C) were statistically significantly reduced in response to metformin at the concentration of 10 mmol/L (\* $p < 0.05$ ), and UDP-GlcNAc also at the concentration of 5 mmol/L (\* $p < 0.05$ ).

declined capability of cultured human VSMCs to migrate in response to metformin exposure. Previously, HA-rich pericellular matrix has been found to be a requirement for the migration and proliferation of VSMCs.<sup>36</sup> Furthermore, the accumulation of HA has been shown to promote neointimal thickening as well as atherosclerosis.<sup>15</sup> In contrast, decreased level of HA has been shown to lead to the development of a less proatherosclerotic phenotype (eg, reduction in the migration and proliferation of human aortic SMCs in vitro).<sup>45</sup>

In addition to the activity of HASEs, the amount of their UDP-sugar substrates has been shown to regulate the rate of HA synthesis.<sup>14</sup> As UDP-sugars use intermediates of glycolysis as starting points (glucose-6-phosphate and fructose-6-phosphate), it can be assumed that metformin might affect their synthesis rate. Furthermore, it has been shown that the rate-limiting enzyme of UDP-GlcNAc



production, glutamine:fructose-6-phosphate amidotransferase 1 activity, is regulated by AMPK.<sup>46</sup> As expected, after metformin treatment, VSMCs exhibited a marked decrease in their cytosolic UDP-sugar pools. Particularly, the level of UDP-GlcNAc was reduced to a quarter within 48 hours with metformin concentration of 10 mmol/L. Although also other mechanisms are likely to be involved, this response of metformin on HA metabolism has not been previously shown.

Just recently, Hao and colleagues revealed that metformin-induced activation of AMPK inhibited proliferation and migration of primary human aortic SMCs via the upregulation of p53 and interferon-inducible protein 16.<sup>47</sup> Another study using human umbilical vein endothelial cells (EC) demonstrated that EC proliferation and apoptosis were regulated through AMPK/cyclic AMP response element-binding protein/brain-derived neurotrophic factor pathway.<sup>48</sup> These present new signalling pathways behind the suggested properties of metformin as an antiatherosclerotic drug. Regarding angiogenesis, metformin-treated human umbilical vein ECs have been reported to exhibit markedly inhibited cell proliferation, migration and decreased expression of matrix metalloproteinases 2 and 9.<sup>49</sup> Also, the decreased formation of capillary-like networks by ECs after metformin treatment has been demonstrated.<sup>50</sup> Both of these antiangiogenic effects of metformin on ECs were shown to be partially AMPK dependent.<sup>49 50</sup>

The pleiotropic effects of metformin extend from the reduction of cardiovascular events to a promising action as an antitumorigenic drug.<sup>51</sup> Indeed, studies suggest that metformin treatment decreases the incidence of cancer among patients with type 2 diabetes.<sup>52 53</sup> In tumor angiogenesis, metformin has been shown to markedly inhibit vascular endothelial growth factor (VEGF) secretion in breast cancer cells.<sup>54</sup> In addition to cancer cells, metformin is able to target the cells in the tumor microenvironment (eg, human white adipose tissue (WAT) cells) and to regulate their gene expression.<sup>55</sup> In a coculture of breast cancer cells together with WAT progenitor cells, treatment with metformin was shown to decrease the amount of several angiogenic proteins including VEGF.<sup>55</sup> In pancreatic cancer, metformin has been revealed to reduce the expression of several fibrogenic cytokines from cancer cells including connective tissue growth factor and TGF- $\beta$ 1 leading to suppression of fibrosis and ultimately to reduction of desmoplastic reaction.<sup>56</sup> Metformin has also been shown to reduce the amount of ECM macromolecules, specifically HA, in pancreatic cancer.<sup>57</sup> This has further been demonstrated to alleviate ECM remodelling and epithelial to mesenchymal transition (EMT).<sup>57</sup> Similarly, decreased fibrosis and EMT associated with reduction in vascular remodelling in the heart and cardiovascular system has been evident after metformin treatment.<sup>7</sup>

Regarding the concepts of programming and epigenetic perspectives, metformin is thought to impact epigenetic programming through decrease in different histone modifications, such as methylation.<sup>53</sup> Also effects on DNA methylation by metformin have been reported.<sup>58</sup> Currently, the long-term effects of gestational metformin treatment are unknown.<sup>59</sup> In our study, gestational metformin treatment caused structural changes in offspring mice aorta wall structure by decreasing the amount of HA. The structural

changes are likely effects of chronic metformin exposure during the gestational stage of offspring.<sup>18</sup>

Our study is confronted with specific limitations in the experimental settings including the restricted sample size of aorta tissues in vivo. However, taken into consideration that only small deviation in the results regarding the offspring aortas was found, the results can be perceived reliable and no larger sample size is needed within this study setting. Furthermore, the results on HA in vivo and in vitro are in complete concordance. The mice tissues were analysed in adulthood (20 weeks of age), excluding the detection of possible alterations associated with offspring age. This was done with the focus on determining the possible long-term programming effects of gestational metformin use. Furthermore, the selected concentrations of metformin for the in vitro experiments may seem high at 1, 5, and 10 mmol/L. However, these were adopted after preliminary experiments and a careful review of previous in vitro studies.<sup>54 60 61</sup> Regarding clinically relevant metformin levels, it has to be taken into account that therapeutic plasma concentration of metformin of patients with type 2 diabetes varies greatly although the dosage guide depicts a variation of 1–3 g/d.<sup>62</sup> Indeed, the concept of ‘therapeutic concentration of metformin’ among patients with type 2 diabetes has been reviewed recently with altogether 120 publications, where the values ranged from 0.129 to 90 mg/L, and the lowest and highest boundaries were 0 and 1800 mg/L (equal to 10.91 mM).<sup>63</sup> This places the metformin concentrations in our in vitro study within the estimated boundaries.

To summarize, in this study gestational metformin use of mice in vivo was shown to be able to influence the composition of the aortic wall of their offspring. Specifically, the content of HA in the aortas of offspring was decreased in response to gestational metformin use. However, there was no change in the intima-media thickness of the aortas of the offspring. It was also shown that the decreased production of HA by cultured human VSMCs in vitro in response to metformin paralleled with the reduction of *HAS2* expression and the depletion of HAS substrates, particularly UDP-GlcNAc. Considering that gestational metformin use seems to be able to program reduced HA content in the vascular wall of the offspring, the data suggest the idea that metformin possesses long-term vasculoprotective effects. Nevertheless, further studies are required to evaluate the safety and long-term effects of gestational metformin. Also clinical trials to examine the long-term vasculoprotective effects of metformin in offspring of women with history of gestational diabetes are needed.

**Acknowledgements** The authors thank Erica Nyman and Eija Rahunen for technical assistance in sample histology processing.

**Contributors** AS and HJ contributed to the study design, manuscript preparation and drafting the manuscript. AS, PT, SO, and SPS contributed to the data collection, analysis and revising the manuscript for important content. HSM, MK, and MS contributed to the study design and data collection. All authors read and approved the final manuscript.

**Funding** The work was supported by the Research Funds of Turku University and University Hospital, Satakunta Central Hospital, and Aarne Koskelo Foundation.

**Competing interests** None declared.

**Patient consent for publication** Not required.

**Ethics approval** The study scheme was approved by the Finnish Animal Experiment Board.

**Provenance and peer review** Not commissioned; internally peer reviewed.

**Data availability statement** All data relevant to the study are included in the article or uploaded as supplementary information.

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