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Sex-specific metabolic and functional differences in human umbilical vein endothelial cells from twin pairs

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HIGHLIGHTS

- No main sex differences were observed in untreated cells.
- Greater VEGF-induced migration in female HUVECs.
- Higher ATP and metabolite levels in female HUVECs after serum starvation and stimulation with VEGF.
- Decrease in ATP levels in male cells after serum starvation.

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ABSTRACT

Background and aims: Gonadal hormones are mainly thought to account for sex and gender differences in the incidence, clinical manifestation and therapy of many cardiovascular diseases. However, intrinsic sex differences at the cellular level are mostly overlooked. Here, we assessed sex-specific metabolic and functional differences between male and female human umbilical vein endothelial cells (HUVECs).
Methods: Cellular metabolism was investigated by bioenergetic studies (Seahorse Analyser) and a metabolomic approach. Protein levels were determined by Western blots and proteome analysis. Vascular endothelial growth factor (VEGF)-stimulated cellular migration was assessed by gap closure. HUVECs from dizygotic twin pairs were used for most experiments.
Results: No sex differences were observed in untreated cells. However, sexual dimorphisms appeared after stressing the cells by serum starvation and treatment with VEGF. Under both conditions, female cells had higher intracellular ATP and metabolite levels. A significant decline in ATP levels was observed in male cells after serum starvation. After VEGF, the ratio of glycolysis/mitochondrial respiration was higher in female cells and

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migration was more pronounced.
Conclusions: These results point to an increased stress tolerance of female cells. We therefore propose that female cells have an energetic advantage over male cells under conditions of diminished nutrient supply. A more favourable energy balance of female HUVECs after serum starvation and VEGF could potentially explain their stronger migratory capacity.

1. Introduction

Underlying reasons for the observed sex differences in risk factors and pathogenesis of cardiovascular diseases are largely unknown [1–4]. Experimental studies have so far focused primarily on the role of sex steroids. However, research in recent years revealed that cells themselves show sex-specific differences [5,6]. The importance to consider the sex of the cells in basic research has been lately emphasized [7–9]. Human umbilical vein endothelial cells (HUVECs) are a widely used model to address cardiovascular disease-related questions in basic research. However, the sex of the cells is usually not taken into account or even known.

Endothelial cell motility plays an important role in the vascular system and represents a prerequisite for angiogenesis and repair of vascular injuries [10,11]. We found a higher ability of female cells to form capillary-like structures compared to male cells, and observed sexual dimorphisms in gene expression in HUVECs [12]. A higher tube formation in female HUVECs was described under hyperoxic conditions [13]. In addition, increased migration was observed in female HUVECs compared to males in untreated cells [14,15]. However, it is not known whether sex-specific differences exist in endothelial cell motility under pro-migratory conditions. Angiogenesis is mainly mediated by vascular endothelial growth factor (VEGF) [16]. Therefore, in the present study we aimed to elucidate sexual dimorphisms in VEGF-mediated cell migration. Since cellular motility is characterized by enhanced energy expenditure, we examined potential sex-specific metabolic differences. It was recently shown that endothelial cells rely mainly on glycolysis during cellular activation and migration [17]. We therefore studied mitochondrial respiration and glycolytic rates in male and female HUVECs. In an approach to reduce biological variability, we also isolated male and female HUVECs from dizygotic twin pairs.

2. Materials and methods

A more detailed description of the methods is provided in the Supplemental data.

2.1. Isolation and cultivation of HUVECs

HUVECs were isolated as previously described [12]. For culture of HUVECs from dizygotic twin pairs 50 ng/ml amphotericin B (Biochrom/Merck; Berlin, Germany) was added to the medium. Isolation of HUVECs conformed to local university guidelines and with the principles outlined in the Declaration of Helsinki. Isolation of male and female HUVECs from dizygotic twin pairs of the opposite sex was approved by the Charité University Hospital Ethics Committee (EA2/017/13) and the mothers provided their written informed consent. Data of the mothers and newborns were provided previously [18]. Experiments with corresponding male/female HUVECs from one twin pair were always performed in parallel and at the same time. Determination of the cellular sex was done as described [12], with the exception that the PCR TaqMan Gene Expression Assays from Applied Biosystems were used: SRY Hs00976796, XIST Hs0179824, beta-actin as reference gene Hs01060665 (Thermo Fisher Scientific, Waltham, MA, USA).

2.2. Migration asssay

Cell migration was induced as recently described [19]. In brief, HUVECs were serum starved in M199 with 0.5% FCS for 5 h in IBIDI culture inserts for migration (Ibidi GmbH, Martinsried, Germany) and treated with either 2 nM human recombinant vascular endothelial growth factor 165 ($VEGF_{165}$, #676472, Merck Chemicals, Darmstadt, Germany) or 5% FCS for 16 h. Cells with 0.5% FCS served as control (starvation). Images were acquired with a Zeiss AxioCam MRm Rev3 camera on a Zeiss Axio ObserverZ.1 microscope (Zeiss, Jena, Germany) equipped with AxioVision 4.8.2 software. For acquisition of videos, photos were taken with a Live Imaging System (Ibidi GmbH, Martinsried, Germany) every 5 min for 36 h. Videos were assembled using Windows Movie Maker.

2.3. VEGFR pathway and downstream proteins

A detailed description of the methods for the activation of the VEGFR pathway and subsequent downstream proteins can be found in the Supplemental data.

2.4. Proteome analysis

HUVECs were grown in 6-cm plates in M199 with 20% FCS. At confluency, cells were scratched with the upper side of a 1000 µl pipette tip, washed twice with M199 containing 0.5% FCS, serum starved with 0.5% FCS for 5 h, followed by treatment with 2 nM VEGF for 16 h. Serum starved cells with 0.5% FCS served as controls. After treatment, cell culture supernatants were collected and centrifuged at 1000 × *g* for 5 min. Adherent cells were washed twice with ice-cold PBS, trypsinized, and cell pellets were stored at −80 °C until analysis. Detailed methods for the proteome analysis are given in the Supplemental data.

2.5. Metabolic assay and determination of intracellular ATP levels

1.5×10^4 cells were seeded in M199 with 20% FCS in XF96 polystyrene tissue culture plates (Seahorse Bioscience/Agilent, Waldbronn, Germany). After 24 h, HUVECs were serum starved with 0.5% FCS for 5 h, followed by treatment with either 2 nM VEGF, 6 nM human recombinant basic fibroblast growth factor (bFGF, #GF003, Merck Chemicals, Darmstadt, Germany) or 20% FCS for 16 h. Serum starved cells with 0.5% FCS served as controls. Non-stimulated cells in M199 with 20% FCS were included as baseline (untreated). Before extracellular flux analysis, plates were incubated in DMEM assay medium containing 0.5% FCS, 2 mM glutamine, 5.5 mM glucose, and 2 mM pyruvate for 1 h at 37 °C in a non-CO_2 incubator. VEGF and bFGF were included in the assay medium during this incubation for 1 h. OCR (oxygen consumption rate) and ECAR (extracellular acidification rate as parameter for glycolysis) were measured in an XFe 96 extracellular flux analyzer (Seahorse Bioscience/Agilent, Waldbronn, Germany). 0.3 µM Oligomycin, 1 µM carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and 0.3 µM Rotenone/Antimycin A were applied in the mitochondrial stress test. Cellular protein levels were measured by Pierce BCA assay and data were normalized to protein content. All values represent the mean from six Seahorse wells.

For measurements of intracellular ATP levels, HUVECs were grown in 6-cm plates in M199 with 20% FCS until confluency, scratched with the upper side of a 1000 µl pipette tip to create a gap, and treated as above. Subsequently, cells were washed with ice-cold PBS and lysed with the ATP assay buffer provided in the kit below. Lysates were centrifuged at 4 °C for 10 min at 15,700 × *g* and supernatants were

deproteinized with TCA using the Deproteinizing Sample Preparation Kit (ab204708, Abcam, Cambridge, UK). After neutralization, samples were stored at −80 °C for short-term. The fluorometric ATP Assay Kit (ab83355, Abcam, Cambrigde, UK) was used according to the instructions of the manufacturer. Measurements were done in a microplate reader at 535/587 nm (excitation/emission).

2.6. GC-MS metabolomics measurement of key central carbon pathway metabolites

HUVECs were serum starved with 0.5% FCS for 5 h, followed by treatment with 2 nM VEGF for 16 h. Serum starved cells with 0.5% FCS served as controls. Untreated cells with complete medium were included as baseline. At the end of the experiments, cells were rapidly washed within 20 s with buffer (140 mM NaCl, 5 mM HEPES, pH 7.4, 37 °C) and quenched in 5 ml of ice-cold methanol (50%) containing 2 µg/ml cinnamic acid (for use as an internal standard). Cells were scraped and the methanolic extracts were collected and analyzed by GC-MS. A detailed description of the metabolomics methods is provided in the Supplemental data.

2.7. Statistical analysis

Comparisons of parameters between male and female HUVECs from non-related pregnancies were performed by One-way ANOVA with post hoc Bonferroni correction. Parameters from HUVECs from dizygotic twin pairs of the opposite sex were compared by paired t-tests. All statistical tests were two-sided, with the level of significance accepted at $p < 0.05$. Statistical analysis was performed using SigmaPlot (Systat Software, San Jose, CA, USA) or Prism (v5; GraphPad Software, La Jolla, CA, USA). Data are given as mean ± SEM.

3. Results

3.1. Greater VEGF-induced migration in female cells

Female HUVECs showed a significantly greater gap closure compared to male cells in response to VEGF, whereas treatment with FCS resulted in a similar extent of migration between the sexes (Fig. 1A). Male cells exhibited lower VEGF-induced migration over the entire time course of 36 h (Supplemental movie files 1 and 2). Female HUVECs showed a significantly greater VEGF-induced gap closure in medium with and without phenol red, indicating that estrogenic activity of phenol red [20] is rather not involved. No sex differences were observed after stimulation with FCS in both media (Fig. 1B).

3.2. Sex differences in the VEGFR pathway and pro-migratory proteins

We observed no significant differences in VEGFR2 Tyr1175 phosphorylation levels between the sexes after treatment with VEGF (Supplemental Fig. 1A). Proximity ligation assays (PLA) revealed no differences in VEGFR2 total tyrosine phosphorylation levels between male and female cells (unstimulated/stimulated phosphorylation 20.2 ± 6.5/29.3 ± 10.1 RCPs/cell for male, and 22.4 ± 7.2/27.4 ± 9.3 RCPs/cell for female HUVECs, n = 5 per sex). Representative images are shown in Supplemental Fig. 1B. Moreover, no distinct sex-specific differences in phosphorylation of downstream kinases were observed (Supplemental Fig. 1C). Basal expression of total VEGFR2 was donor-dependent and, as reported [21], decreased after long-term treatment with VEGF, independently of the cellular sex. Levels of other proteins known to be involved in migration, like vinculin, α-catenin, and moesin, were not affected by treatment with VEGF for 16 h (Supplemental Fig. 2A). Levels of secreted VEGFR1, a negative regulator of cell migration by competitive binding to VEGFA [22], were strongly increased by VEGF. However, no significant sex differences were observed (Supplemental Fig. 2B).

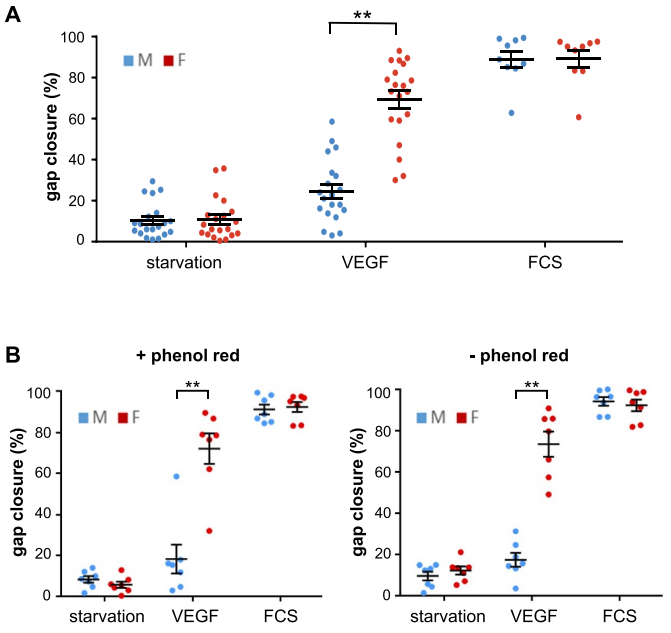

Fig. 1. Greater VEGF-induced migration in female (F) compared to male (M) HUVECs.
Cells were serum starved for 5 h in 0.5% FCS in IBIDI migration chambers and subsequently treated with 2 nM VEGF or 5% FCS. Serum starved cells with 0.5% FCS served as controls. Photos were taken at the beginning and after 16 h and the percentage of gap closure was quantified using ImageJ software. (A) Significantly greater VEGF-induced migration in female as compared to male HUVECs was observed, whereas no sex differences were seen after treatment with 5% FCS. Data are from n = 20 different donors from each sex (for starvation and VEGF) and from n = 9 for FCS. (B) Significantly greater VEGF-induced migration in female cells was obtained with and without phenol red. No sex differences were detected after treatment with 5% FCS under both conditions. Data are from n = 7 different donors for each sex. $^{**}p < 0.01$ male *vs.* female. Results are mean ± SEM.

For a comprehensive insight into sex-specific VEGF-induced protein alteration, a global protein profiling was performed. To reduce inter-individual biological variability, male and female HUVECs from dizygotic twin pairs of the opposite sex were used in this and all subsequent experiments. Proteome analysis revealed 106 intracellular and 26 extracellular proteins with different subcellular locations and functional categories with significant sex-specific differences. Of the 164 quantified proteins of the gene ontolgy (GO) term "endothelial cell migration", 56 intracellular proteins showed significant changes after VEGF compared to control (starvation), 37 in male and 43 in female cells (Supplemental Tables 1 and 2). VEGF resulted in downregulation of ribosomal proteins and of proteins involved in cellular disassembly in male cells, whereas in female cells mitotic cell cycle proteins were downregulated and proteins involved in Golgi vesicle transport were upregulated (Fig. 2A). In addition, increased levels of proteins involved in migration were observed in the cell culture supernatant (Supplemental Table 1, lower part). Levels of secreted endothelin-1 were regulated in the opposite direction by VEGF between the sexes (down in male, up in female) and MMP-10 showed significantly higher levels in female cells (Fig. 2B). Increased endothelial nitric oxide synthase (eNOS) levels were linked with higher migration of female HUVECs in untreated cells [15]. Although we could confirm higher eNOS levels in untreated female HUVECs, we observed no sex differences in eNOS protein levels after treatment with VEGF (male/female ratios: untreated −1.23, $p = 0.06$; VEGF 1.07, $p = 0.33$, n = 15 per sex).

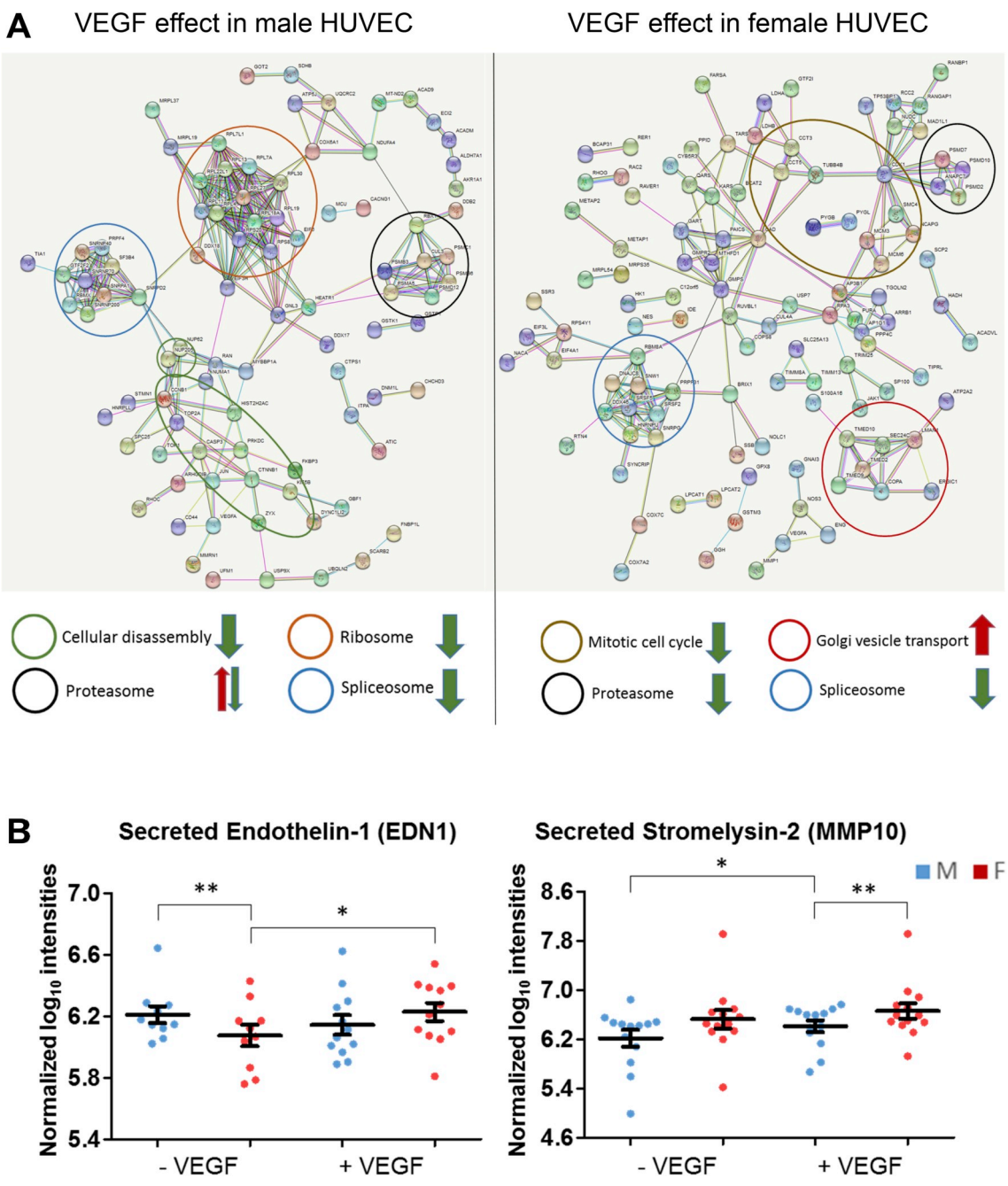

Fig. 2. Differential changes in protein levels in male and female HUVECs from twin cells after treatment with 2 nM VEGF for 16 h compared to serum starvation. (A) Significant sex-specific alterations of intracellular proteins by VEGF were analyzed by STRING and protein clusters annotated accordingly. Green arrows indicate lower abundance and red arrows higher abundance of proteins in the respective sex. Data are from n = 15 male/female twin pairs. (B) Log10 of normalized protein intensities for secreted endothelin 1 and MMP-10. Cells were treated or not with 2 nM VEGF for 16 h. Shown are dot plots with mean ± SEM. Results are from the illustrated number of male/female twin pairs. *$p < 0.05$ between treatments; **$p < 0.05$ between sexes.

3.3. Sexual dimorphisms in cellular energy supply after serum starvation and VEGF

Parameters of mitochondrial respiration like maximal respiration or ATP production did not differ between the sexes under all treatments (Supplemental Fig. 3). However, basal mitochondrial respiration was significantly lower in female HUVECs compared to male cells after VEGF, whereas no significant sex differences were obtained for all other treatments (Supplemental Fig. 4). VEGF resulted in higher mitochondrial respiration in male HUVECs in 14 out of 15 twin pairs (Supplemental Fig. 5). This was not observed in untreated cells or after bFGF. The ratios of proteins of the mitochondrial electron transport chain (complex 1–5) were close to 1 in male and female HUVECs and did not change with treatments (untreated 1.00; serum starvation 0.97; VEGF 1.00; bFGF 1.03; n = 15 male/female twin pairs).

A shift in energy production towards glycolysis was described for endothelial cells during cellular activation and motility [17]. We observed a strong sex-independent increase in glycolysis by serum starvation that was further potentiated by VEGF (Fig. 3A). Ratios of glycolysis versus mitochondrial respiration (ECAR/OCR ratios) revealed no sex differences in untreated cells. However, serum starvation and VEGF resulted in a slightly higher ECAR/OCR ratio in female HUVECs ($p = 0.053$ and $p = 0.227$ for serum starvation and VEGF, respectively) (Fig. 3B). Both conditions resulted in increased ECAR/OCR ratios for

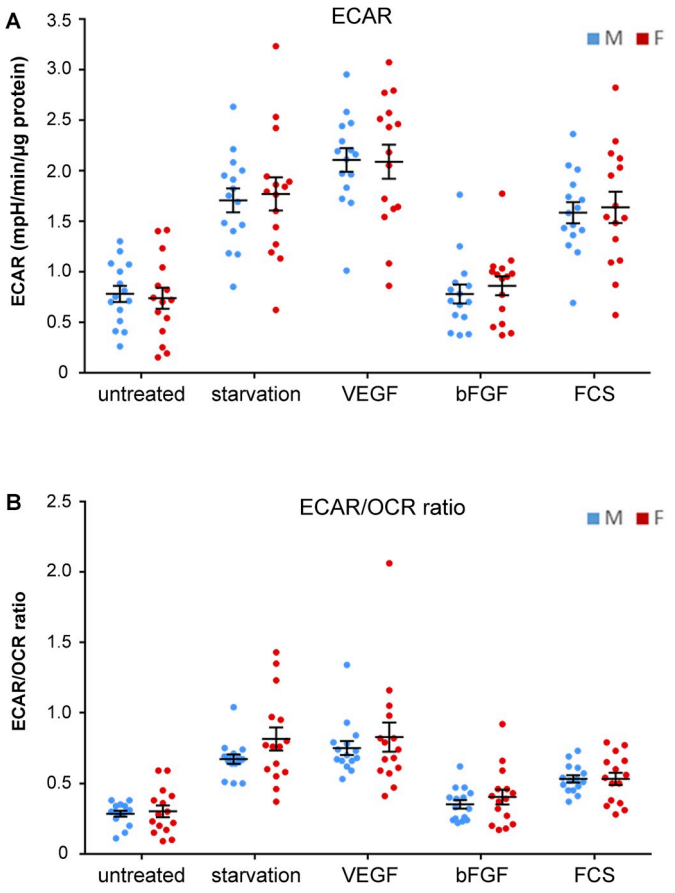

Fig. 3. Rate of glycolysis and mean ECAR/OCR ratios in male (M) and female (F) HUVECs from twin pairs.
Cells were either left untreated, serum starved or treated with 2 nM VEGF, 6 nM bFGF or 20% FCS for 16 h. (A) Glycolytic rate (ECAR) was measured using Seahorse. (B) ECAR/OCR ratios (glycolysis/mitochondrial respiration) are shown for the indicated treatments. All data are mean ± SEM from n = 15 male/female twin pairs.

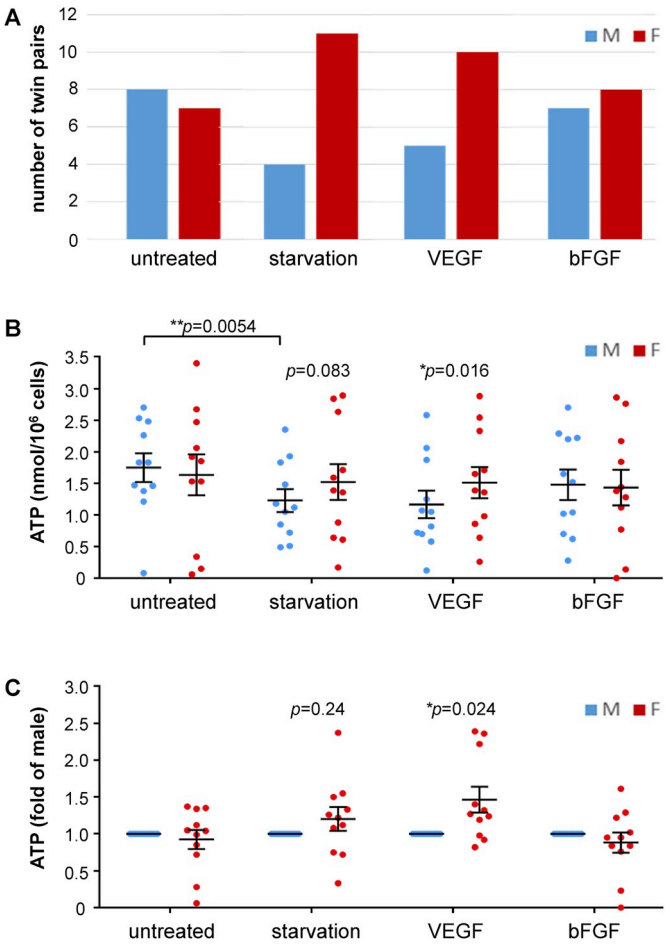

Fig. 4. Increased ECAR/OCR ratios and higher ATP levels in female (F) compared to male (M) HUVECs from twin pairs.
Cells were either left untreated, serum starved, or treated with 2 nM VEGF or 6 nM bFGF for 16 h. (A) The number of twin pairs showing a higher ECAR/OCR ratio (glycolysis/mitochondrial respiration) in either the male or the female cells are shown. (B) Absolute and (C) relative (folds of male) intracellular ATP levels after the indicated treatments. *p*-values indicate statistical comparisons between sexes or treatments. Data are mean ± SEM from n = 11 male/female twin pairs.

female HUVECs in most twin pairs (Fig. 4A).

In untreated cells, slightly higher ATP levels were seen in male HUVECs. In contrast, serum starvation and VEGF resulted in higher ATP levels in female cells, reaching statistical significance for VEGF. Similar ATP levels between the sexes were obtained after bFGF (Fig. 4B and C). Serum starvation resulted in a significant decrease of intracellular ATP levels in male HUVECs, whereas only a minor reduction was observed in female cells (Fig. 4B).

3.4. Higher levels of intracellular metabolites in female HUVECs after serum starvation and VEGF

20 metabolites were annotated in a metabolomics survey. In untreated HUVECs, male cells had a tendency towards slightly higher metabolite levels (Fig. 5A; Supplemental Figs. 6 and 7). In contrast, after serum starvation and VEGF the central carbon metabolite levels of female cells increased. Altogether, 8 and 4 metabolites were significantly higher in female compared to male cells after serum starvation and VEGF, respectively (Fig. 5A). The number of metabolites with differences between female and male cells for the experimental conditions are shown in Fig. 5B. The combined analysis of the metabolites belonging to glycolysis and TCA cycle revealed no sex differences in untreated cells. Amino acid pools were significantly higher in male cells ($p < 0.05$). In contrast, under serum starvation glycolytic as well as TCA-related metabolites, and amino acid pools, all increased significantly ($p < 0.05$) in female HUVECs. In addition, TCA cycle metabolites and amino acids were significantly ($p < 0.05$) higher in female cells after VEGF (Fig. 5B). Compared to untreated cells, serum starvation resulted in significantly higher central carbon metabolite levels in female HUVECs, mainly due to increased amino acid pools (Fig. 5C and D; Supplemental Figs. 8 and 9). VEGF led to an overall lower metabolite pool in male and female HUVECs ($p < 0.01$) compared to serum starvation (Supplemental Fig. 10). An overview of individual metabolite levels in male and female cells across all conditions is shown in Supplemental Fig. 11.

4. Discussion

In our study, we observed no major sex differences under basal conditions (in untreated cells) in cellular energy production (glycolysis versus mitochondrial respiration), and in intracellular ATP and metabolite levels. However, serum reduction and treatment with VEGF led to sexual dimorphisms with higher ATP and metabolite levels in female cells, potentially attributed to a shift in the ECAR/OCR ratio in female HUVECs. The results of the proteome analysis could point to an increased cellular flexibility of female cells and a reduced capacity of male cells for protein synthesis. In sum, these findings could indicate a better energy balance of female cells under stressed conditions as

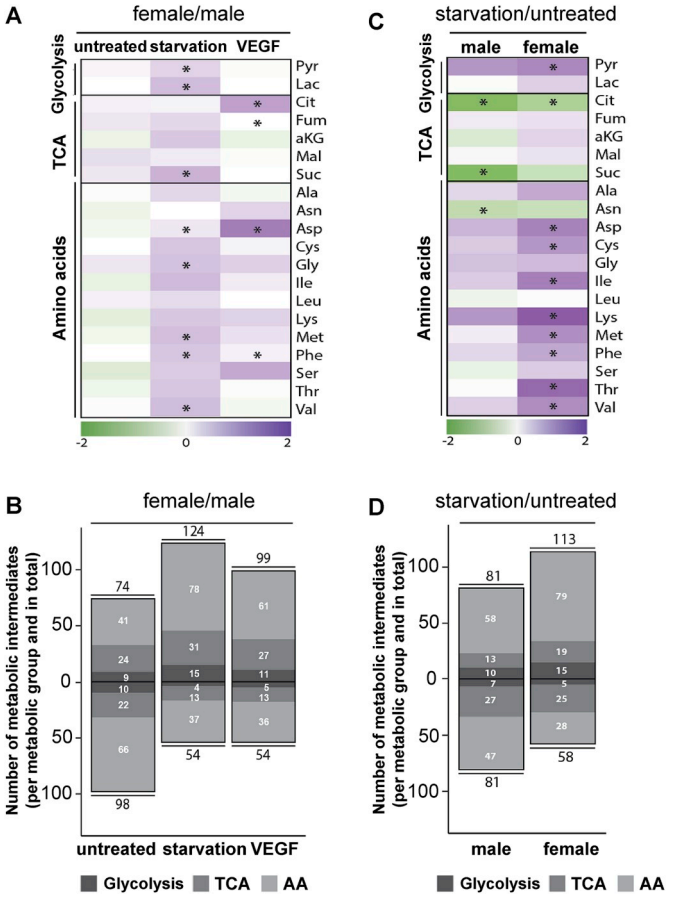

Fig. 5. Levels of intracellular metabolites in male and female HUVECs from twin pairs.

Cells were either left untreated, serum starved, or treated with 2 nM VEGF for 16 h. Heat map of the mean log2 of the female/male (A) or starvation/untreated (C) ratios of normalized peak areas. Metabolites were assigned to the groups of glycolysis, TCA and amino acids. *$p < 0.05$. Number of metabolites (in total and according to the group of glycolysis, TCA and amino acids) after unit scaling of the log2 for female/male (B) or starvation/untreated (D) ratios of normalized peak areas for each twin pair. Metabolites with higher peak areas are shown above, those with lower peak areas below zero. Data are from n = 10 male/female twin pairs for untreated and starvation and n = 9 for VEGF. TCA, tricarboxylic acid; AA, amino acids.

potential underlying mechanism for functional differences. Accordingly, we observed a greater VEGF-induced migration in female HUVECs.

Migration of endothelial cells represents an initial step during formation and repair of blood vessels. VEGF, produced by a number of different cell types, is a major regulator of angiogenesis [23]. In the present study, we observed a greater gap closure in female compared to male cells in response to VEGF. Sex differences in cellular motility have been previously described. In bone marrow-derived circulating endothelial progenitor cells (EPCs), colony-forming capacity and migration was stronger in cells from middle-aged women compared to men [24]. A slightly higher gap closure was observed in female HUVECs compared to males under basal conditions [14], and a better wound closure was described in female aortic rat endothelial cells [25]. Under hyperoxic conditions, female HUVECs showed significantly higher tube formation capacity [13]. Recently, increased endothelial nitric oxide synthase (eNOS) expression and activity was associated with better migration of female HUVECs in untreated cells [15]. However, in our study we observed no sex differences in eNOS expression after treatment with VEGF, indicating that this enzyme is not involved in better VEGF-induced migration of female HUVECs. Furthermore, we found no sex differences in migration in response to FCS.

To elucidate potential underlying mechanisms for these sex differences, we searched for differences in the VEGFR pathway. Although we observed trends for increased VEGFR2 phosphorylation and activation of downstream kinases in female HUVECs, due to substantial variability in protein expression and phosphorylation levels between different donors, data are inconclusive. We therefore focused on male and female HUVECs from twin pairs. These cells are *in vivo* exposed to the same maternal environmental conditions (life style, diet, smoking, alcohol, medication, chronic diseases etc.), and a similar hormonal and inflammatory status during pregnancy. Therefore, they represent a valuable tool to study intrinsic sex-specific differences at the cellular level. In a proteomic approach with HUVECs from 15 twin pairs, we observed a significant impact of VEGF on proteins involved in migration. Levels of secreted endothelin-1 were upregulated in female and downregulated in male cells by VEGF and higher levels of MMP-10 were found in female cells. Since both endothelin-1 and MMP-10 promote VEGF-induced migration and angiogenesis in HUVECs [26,27], sex-specific regulation of these proteins could at least partly contribute to the observed better migration of female cells. Overall, sex differences in protein abundance were rather small. Small sexual dimorphisms in protein levels in HUVECs from twin pairs have also been described recently [18].

Since endothelial migration requires increased energy production [28,29], we compared cellular metabolic activity between male and female endothelial cells. Male cells had a significantly higher mitochondrial respiration after treatment with VEGF. However, endothelial cells rely on glycolysis during cellular activation and migration [17]. Around 80% of the ATP production in endothelial cells is derived from glycolysis [30]. Serum starvation resulted in a sex-independent increase in glycolysis together with a decrease in mitochondrial respiration, reflecting cellular adaptations in energy metabolism in response to a decline in nutritional supply. Importantly, more female than male HUVECs had a higher ECR/OCR ratio after serum starvation and VEGF treatment. Moreover, female HUVECs exhibited also higher intracellular ATP levels under both conditions. This can be attributed either to increased ATP production and/or lower ATP consumption. The observed decline in intracellular ATP levels after serum starvation in male, but not female cells, point to an impaired energy balance in male cells under growth factor deprivation. This is consistent with our earlier findings of decreased cell viability in male cells after serum starvation [12]. Furthermore, other cellular sources of ATP generation than mitochondrial respiration or glycolysis could be involved. The production of ATP in the nucleus was recently described [31]. In agreement with our results, no sex differences in cellular ATP levels were previously observed in untreated male and female HUVECs [32].

In the metabolomics study, a number of amino acids had higher levels in untreated male HUVECs. This is in line with the data from the study by Ruoppolo et al., in which higher levels of asparagine, isoleucine, and methionine were detected in serum of adult men [33]. Serum concentrations of most amino acids were also significantly higher in males in large cohort studies [34,35]. Sex differences in blood metabolites were shown to be associated with the hormonal status [36]. However, the above and our findings of a general higher amino acid pool in males (both in blood and at the cellular level), are suggestive of intrinsic sex-specific differences at the cellular level, regardless of hormonal influences.

The increased metabolite levels in female cells after serum starvation and VEGF might be driven either by higher metabolic activity or, conversely, by a decrease in metabolic activity due to higher rates of metabolite accumulation in the cell. Since our findings show increased intracellular ATP levels and better VEGF- (but not FCS-) induced migration in female cells, they are indicative of a higher or more efficient metabolic activity. This is also supported by higher cell viability of

female cells under serum starvation [12]. Better ability of female cells to cope with nutritional stress could result in a benefit to translate the VEGF signals into migration.

To our knowledge, there are currently no clinical data about sex differences in angiogenesis. However, female mice showed higher angiogenesis in perigonadal adipose tissue after a high-fat diet [37]. Differences between sexes were also reported for wound healing, a process requiring cell migration. An improved outcome for female compared to male children was observed after thermal injury. Females had a lower incidence of severe infections and signs of faster wound healing [38]. *In vivo*, hormones, especially estrogens, are thought to play a role in blood vessel formation and wound healing [39–41]. We observed no effect of phenol red with reported potential estrogenic activity in the cell culture medium [20] on sex differences in VEGF-induced migration patterns. Also, ovariectomy did not reverse the better wound healing of the skin observed in female compared to male mice [42]. In summary, these results suggest that the sex-specific differences in cell motility and wound repair *in vitro* and *in vivo* represent an intrinsic cellular and hormone-independent effect.

Higher intracellular ATP and metabolite levels in female HUVECs after serum starvation and VEGF along with increased VEGF-induced migration, point to intrinsic sex-specific differences at the cellular level. The absence of sex differences in untreated cells indicate that sexual dimorphisms might be more pronounced under stressed conditions.

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Author contributions

Mario Lorenz: wrote the manuscript with help from all other authors, conceived and designed the experiments.

Benjamin Blaschke: performed and analyzed Seahorse experiments.

Andreas Benn: performed and analyzed migration experiments.

Elke Hammer: performed and analyzed proteome experiments.

Eric Witt: performed and analyzed proteome experiments.

Jennifer Kirwan: designed and analyzed metabolomics study.

Raphaela Fritsche-Guenther: performed and analyzed metabolomics study.

Yoann Gloaguen: performed statistical analysis.

Cornelia Bartsch: isolated HUVECs, performed Western blots.

Angelika Vietzke: isolated HUVECs, performed Western blots.

Frederike Kramer: performed and analyzed PLA experiments.

Kai Kappert: designed and analyzed PLA experiments.

Patrizia Brunner: performed and analyzed migration experiments.

Hoang Giang Nguyen: performed and analyzed migration experiments.

Henryk Dreger: coordinated and conceived the collection of cells from twin pairs.

Karl Stangl: edited and critically revised the manuscript.

Petra Knaus: conceived and designed migration experiments.

Verena Stangl: supervised the study, edited and critically revised the manuscript.

Declaration of competing interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.10.007.

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