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Marina C. Seefried [a], Ines Lurtz [a], Johanna Mittelberger [a], Manuela Franitza [a], Jonas Bubmann [a], Carl Mathis Wild [a], Nina Ditsch [a,b], Birgit Urban [a], Christina Kuhn [a], Christian Dannecker [a], Peter Altevogt [c], Udo Jeschke [a,*], Marei Sammar [d,*]

[a] *Obstetrics and Gynecology, Faculty of Medicine, University of Augsburg, Stenglinstrasse 2, Augsburg 86156, Germany*
[b] *Senology, Obstetrics and Gynecology, Faculty of Medicine, University of Augsburg, Stenglinstrasse 2, Augsburg 86156, Germany*
[c] *DKFZ and University Medical Center Mannheim, Ruprecht-Karl University of Heidelberg, Germany*
[d] *Department of Biotechnology Engineering, Braude College of Engineering, Karmiel, Israel*

A R T I C L E I N F O

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A B S T R A C T

New data indicate an increased risk of obstetric complications after Covid-19, the aim of this study was therefore to investigate the expression of immune checkpoint molecules TIM-3 and Gal-9 in tissue sections of placenta from patients with acute Covid-19, from patients after Covid-19 infection and from healthy controls. We used a total of 60 placentas from women who had given birth to female or male children at Augsburg University Hospital, Germany. We examined 10 placentas each with acute Covid-19 from female and male fetuses, 10 placentas each from female and male fetuses after acute Covid-19 and 10 placentas each from female and male fetuses of a healthy control group. Immunohistochemical staining against TIM-3 and Gal-9 was performed, and marker expression was assessed by an immunoreactive score (IRS). The identity of cells expressing TIM-3 or Gal-9 was then analyzed by double immunofluorescence analysis. TIM-3- and Gal-9 positive macrophages are significantly upregulated in the decidua of female acute Covid-19 placentas. TIM-3 is significantly up-regulated in male extravillous trophoblast cells in the acute phase of Covid-19. On the other hand, TIM-3 was significantly downregulated in Hofbauer cells from male placentas after Covid-19. Gal-9, the ligand for TIM-3, was significantly downregulated in Hofbauer cells from male placentas after Covid-19. Triple immunofluorescence analysis showed that male acute Covid-19 Hofbauer cells (analyzed by CD163 staining) were positive for both TIM-3 and Gal-9. Surprisingly, the observed effects were related to fetal sex. In particular, the formative effect of this infection needs to be analyzed in further studies.

1. Introduction

When the Covid-19 pandemic began, there was a great deal of uncertainty and fear, especially among pregnant women, due to the uncertainty about the virus itself and its possible dangers during pregnancy (Meister et al., 2023). This increased anxiety has been confirmed in several studies (Hagenbeck et al., 2020, 2023; Schaal et al., 2023, 2022). Since pregnancy is associated with a general immunomodulation of the pregnant woman, but not necessarily with an immunocompromised state, pregnant women are more susceptible to viral infections due to the immune changes. During the covid-19 pandemic, pregnant women were more likely to suffer from severe disease (Accurti et al., 2022). An increased mortality rate in pregnant women with Covid-19 infection has also been observed in other studies (Accurti et al., 2022; Ahmad et al., 2022; Ambedkar et al., 2023). New evidence suggests an increased risk of obstetric complications, including maternal complications such as gestational diabetes or hypertension or fetal complications such as premature birth, intrauterine growth restriction, stillbirth or the risk of developmental disorders in the newborn. Overall, there remain conflicting concerns about the potential risk of vertical transmission of infection to the fetus (Tosto et al., 2023).

In addition, early studies suggested that residual Covid-19 and immune responses persist in the placenta of pregnant patients who have recovered from Covid-19 infection (Wu et al., 2021). In the same study, infiltration of CD14 + macrophages into the placental villi of pregnant women who had recovered from Covid-19 infection was higher than in

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* Corresponding authors.
E-mail addresses: udo.jeschke@med.uni-augsburg.de (U. Jeschke), sammar@braude.ac.il (M. Sammar).

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normal control pregnancies (Wu et al., 2021). In addition, another study described a different subcellular localization pattern of viral RNA in alveolar type 2 cells and alveolar macrophages of pregnant women (Acheampong et al., 2021). In a study by Juttukonda et al., the decidual immune response to Covid-19 was investigated during pregnancy (Juttukonda et al., 2022a). It showed a small but significant difference in CD14 staining when comparing male and female infants in the control groups. This could not be replicated in the Covid-19 groups (Juttukonda et al., 2022a). Another group divided 29 placentas from 29 SARS-CoV-2-positive mothers into two groups according to symptomatology (moderate/severe vs. asymptomatic/mild) and performed immunohistochemistry for CD4 + and CD8 + T lymphocytes and for CD68 + macrophages (Resta et al., 2022). They found a massive increase in CD68-positive macrophages, particularly in the placentas of intensive care patients (Resta et al., 2022). In another study by Juttukonda et al., the effects of maternal SARS-CoV-2 vaccination and first trimester infection on the feto-maternal immune response were investigated (Juttukonda et al., 2022b). They found no changes in the amount of decidual macrophages in vaccinated patients compared to controls (Juttukonda et al., 2022b). More recently, a group led by Barrozo et al. investigated the SARS-CoV-2 niches in the human placenta using spatial transcriptomics data (Barrozo et al., 2023). They were able to show a significant decrease in the proportion of M2 polarized macrophages and a significant increase in M0 to M1 transition macrophages at the gene level in the highly positive SARS-CoV-2 placental samples (Barrozo et al., 2023). In addition, our own investigations found that in opposite to PE (Mittelberger et al., 2023), there is an upregulation of CD163-positive decidual macrophages of male acute Covid-19 placentas (Seefried et al., 2024). We found a significantly overexpression of PD-L1 in male acute and post Covid-19 decidual cells, as well as in male post Covid-19 extravillous trophoblast cells (Seefried et al., 2024). We also showed a significant downregulation of CD24 expression on extravillous trophoblasts and Hofbauer cells in female acute Covid-19 placentas (Seefried et al., 2025). In contrast, CD24 is significantly upregulated on male post covid-19 Hofbauer cells (Seefried et al., 2025). The CD24 ligand Siglec-10 is significantly downregulated in Hofbauer cells after Covid-19 infection regardless of fetal sex, while it is significantly more highly expressed in female Hofbauer cells (Seefried et al., 2025). CD24 and its ligand Siglec-10 are differentially expressed in the placenta of patients who have survived a Covid-19 infection (Seefried et al., 2025). Finally, the group of Altunaş et al. attempted to decipher the effects of Covid-19 on pregnancy from the perspective of placental histopathology and umbilical cord macrophage immune activity, as well as by comparison with neonatal outcomes (Altuntas et al., 2024). Using a mouse macrophage marker (F8/80), they found a significant increase in the number of macrophages in Warton's jelly and a significantly increased ratio of macrophages to other cells (Altuntas et al., 2024).

Immune checkpoint molecules, such as T-cell immunoglobulin and mucin-domain containing protein 3 (TIM-3) and its ligand Galectin-9 (Gal-9), play a crucial role in maintaining immunological homeostasis and maternal-fetal tolerance. Upon activation by their ligands, these molecules primarily exert inhibitory effects on the immune system by downregulating effector cells like T cells. As such, they are key regulators in various contexts, including tumor progression, autoimmunity, and infectious diseases (Kandel et al., 2021). Beyond these roles, TIM-3 and Gal-9 are also essential in reproductive immunology and the maintenance of pregnancy (Mittelberger et al., 2022; Mohamed Khosroshahi et al., 2021). Emerging evidence indicates that alterations in these molecules are associated with pregnancy complications such as preeclampsia (Mittelberger et al., 2024). However, while most existing research focuses on their expression in peripheral blood, little is known about their presence and function in the placenta and at the maternal-fetal interface (Mittelberger et al., 2022), specifically in Covid-19 placentas.

In the present study, we analyzed TIM-3 and Gal-9 protein levels in placental tissue from acute Covid-19 patients, from patients who survived Covid-19 infection and from normal term controls. As there are no sufficient studies on the expression of TIM-3 and its ligand Gal-9 to date, these analyses aimed to investigate both TIM-3 and Gal-9 in acute and post Covid-19 placental tissue, with a particular focus on TIM-3/ Gal-9 expressing cell populations associated with Covid-19 infection.

2. Materials and methods

2.1. Study subjects

This study was approved by the Ethics Committee of the Ludwig-Maximilian-University (LMU) Munich, Germany, in July 2021. Placental tissue from 60 placentas, 10 placentas each with acute Covid-19 infection from female and male fetuses, 10 placentas each after Covid-19 from female and male fetuses and 10 placentas each from healthy mothers who gave birth to a female or male fetus at Augsburg University Hospital in the years 2020–2022 were obtained after written informed consent and included in the study. The control group was matched to the Covid-19 and post Covid- 19 study group in terms of week of pregnancy, fetal sex and maternal age (+/- 5 years). To exclude confounding factors, patients who met the following criteria were excluded from the study: Preeclampsia, HELLP, intrauterine growth restriction, fertility treatment, signs of systemic inflammation in the blood other than Covid-19, placental disorders such as placenta accreta/ percreta/increta. In addition, all groups were non-vaccinated. Post Covid-19 patients had a positive rapid test and a positive PCR test during pregnancy but were asymptomatic at the time of delivery.

Immediately after delivery the placentas were placed in buffered formalin for preservation. The samples were taken from the placenta's central part at the Institute of Pathology, Augsburg University Hospital. They contained decidua, extravillous and villous trophoblasts. After fixation in buffered formalin, the samples were embedded in paraffin and cut into 2–3μm thick slices using a sliding microtome.

The clinical details of the study population are shown in Table 1.

2.2. Immunohistochemistry

For immunohistochemistry, we deparaffinized the paraffin sections with Roticlear® before immersing them in 100 % ethanol. The endogenous peroxidase activity was blocked by an incubation in 3 % H2O2 in methanol for 20 min and the samples were then rehydrated in a descending alcohol gradient to distilled water. Then, the slices were placed in a high-pressure cooker for 5 min using boiling sodium citrate buffer at pH 6.0 to retrieve the antigen.

Next followed a treatment of the slices with a blocking solution for five minutes. This was Reagent 1 from the ZytoChem Plus134 HRP Polymer System IgG kit (Mouse/Rabbit) by Zytomed, which saturates electrostatic charges. Then the tissue sections were incubated for 16 h at 4 °C with TIM-3 or 45 min at room temperature with Gal-9. The slides were then washed with phosphate-buffered saline (PBS). Finally, the ZytoChem Plus HRP Polymer System IgG kit (Mouse/Rabbit) (Zytomed, Berlin, Germany) and liquid DAB+ (Diaminobenzidin) Substrate Chromogen System (Agilent Technologies, Santa Clara, USA) was used to visualize the bound primary antibodies. The slices were counterstained with Mayer's acid hemalum for 2 min and stained blue for 5 min in tap water. The next step was to dehydrate the samples in an ascending series of alcohol and treat them with Roticlear®. Finally, cover-slipe them with RotiMount (Carl Roth, Germany).

All antibodies which were used in this study are listed in Table 2.

2.3. Microscopy imaging and analysis techniques

The staining was assessed and evaluated with a digital microscope (Keyence, Osaka, Japan). The number of antigen-presenting macrophages and Hofbauer cells was assessed by counting the number of cells in three sections at 40x magnification. The total number of cells was

Table 1
Clinical details of the study population.

	Control (n = 20)	Acute Covid (n = 20)	Post Covid (n = 20)	p-value
	Male (n = 10)	Male (n = 10)	Male (n = 10)	
	Female (n = 10)	Female (n = 10)	Female (n = 10)	
Age at delivery	32,26 ± 3,49	31,26 ± 5,31	30,95 ± 4,14	p = 0,57
	32,00 ± 3,16	30,60 ± 5,76	31,50 ± 3,78	p = 0,66
	32,50 ± 3,92	31,67 ± 5,07	30,40 ± 4,60	p = 0,68
BMI (before pregnancy)	24,90 ± 4,81	25,43 ± 4,85	25,47 ± 5,18	p = 0,82
	25,96 ± 4,48	25,84 ± 4,64	25,75 ± 4,52	p = 0,99
	23,94 ± 5,12	24,98 ± 5,31	25,18 ± 6,00	p = 0,72
Gravidity	1,79 ± 1,08	2,32 ± 1,00	2,25 ± 1,33	p = 0,27
	1,78 ± 0,97	2,00 ± 0,82	2,10 ± 1,37	p = 0,94
	1,80 ± 1,23	2,67 ± 1,12	2,40 ± 1,35	p = 0,17
Parity	1,37 ± 0,50	2,05 ± 0,71	2,05 ± 1,28	**p = 0,02**
	1,44 ± 0,53	1,80 ± 0,63	2,10 ± 1,37	p = 0,55
	1,30 ± 0,48	2,33 ± 0,71	2,00 ± 1,25	**p = 0,02**
Gestational age at delivery	39,25 ± 1,92	39,20 ® 2,02	39,25 ± 2,10	p = 0,99
	39,40 ± 1,90	39,30 ± 2,11	39,40 ± 1,90	p = 1,00
	39,10 ± 2,03	39,10 ± 2,03	39,10 ± 2,38	p = 0,98
Birthweight (g)	3178,95 ± 506,60	3268,68 ± 404,91	3165,00 ± 627,41	p = 0,96
	3343,78 ± 409,12	3279,50 ± 422,84	3375,00 ± 594,61	p = 0,49
	3030,60 ± 559,36	3256,67 ± 409,21	2955,00 ± 616,00	p = 0,80
APGAR 10 min	9,89 ± 0,32	9,74 ± 0,56	9,95 ± 0,22	p = 0,14
	9,78 ± 0,44	9,60 ± 0,70	10,00 ± 0,00	p = 0,19
	10,00 ± 0,00	9,89 ± 0,33	9,90 ± 0,32	p = 0,33
Umbilical artery pH	7,26 ± 0,11	7,27 ± 0,09	7,28 ± 0,07	p = 0,58
	7,25 ± 0,11	7,25 ± 0,11	7,28 ± 0,07	p = 0,59
	7,26 ± 0,12	7,29 ± 0,06	7,28 ± 0,08	p = 0,89

Table 2
List of primary antibodies for immunohistochemistry/immunofluorescence.

Antibody	Isotype	Clone	Dilution	Source
Anti-TIM−3	Rabbit IgG	Monoclonal; clone D5D5R	1:150	Cell Signaling, USA
Anti-Gal−9	Mouse IgG2B	Monoclonal; clone 1005401	1:300	Cell Signaling, USA
Anti-CD163	Mouse IgG1	Monoclonal; clone OTI2G12	1:2000	Abcam, UK
Anti-CK7	Mouse IgG1, kappa	Monoclonal; clone OV-TL 12/30	1:200	Agilent, USA

then calculated by summing the three areas. To evaluate the intensity and distribution patterns of antigen expression in the extravillous trophoblast and the syncytiotrophoblast, we utilized the semi-quantitative immunoreactive score of Remmele (IRS) (Kandel et al., 2021; Mittelberger et al., 2022). To calculate the IRS, we multiplied the grade of optical staining intensity (0 =none, 1 =weak, 2 =moderate and 3 =strong staining) by the percentage of positive staining cells (this was divided into 4 categories: 0 =none, 1 =weak, 2 =moderate and 3 =strong staining): 0 =no staining, 1 = <10 % of the cells, 2 = 10–50 % of the cells, 3 = 51–80 % of the cells and 4 =more than 80 % of the cells).

2.4. Immunofluorescence

The double immunofluorescence staining method was used to characterize specific antigens simultaneously. We then placed the same formalin-fixed and paraffin-embedded samples in Roticlear® for 20 min for deparaffinization. Afterwards the sections were immersed in descending concentrations of ethanol (100 %, 70 %, 50 %) and rinsed in distilled water. Antigens were retrieved by heat pre-treatment for 5 min in a pressure cooker with EDTA buffer, pH 9.0. Then, the sections were washed in distilled water and PBS for 4 min. Next, incubation with immunofluorescence blocking buffer (Cell Signaling; USA) was performed to prevent unspecific staining. After 60 min, the solution was dispensed. The primary antibodies listed in Table 2 were applied at 4 °C for 16 h or 45 min at 22 °C in the case of Gal-9. Another washing step with PBS followed, then the bound primary antibodies were marked by using secondary antibodies (Table 3) at room temperature for 30 min.

Sections were again washed in PBS, covered with True Black for 1 min at 22°C to suppress tissue autofluorescence, and then washed again. After drying, the slides were cover slipped using DAPI containing fluorescence mounting medium (Vector Laboratories; USA). The quadruple staining was assessed and evaluated with a fluorescence microscope (Keyence, Osaka, Japan).

2.5. Statistics

Statistical analysis was performed using SPSS version 24 (SPSS Inc., Chicago, IL, USA). All variables were assessed for normal distribution using the Shapiro-Wilk test. As the data was non-normal, non-parametric tests were used. We used the Kruskal-Wallis test to compare the differences in all groups (acute, post-acute and non-acute). We further stratified each group by fetal sex (male and female). For pairwise comparisons between two specific groups, Mann–Whitney U tests were applied. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Expression of TIM-3 in the extravillous trophoblast

Analyses of TIM-3 in the extravillous trophoblast (EVT) revealed that

Table 3
List of secondary antibodies for immunofluorescence.

Target Antibody	Detection antibody	Dilution	Source
Anti-CD163	Goat-anti-mouse IgG, Cy2	1:100	Jackson Immunotech, USA
Anti-CK7	Goat-anti-mouse IgG, Cy2	1:100	Jackson Immunotech, USA
Anti-TIM−3	Goat-anti-rabbit IgG; Cy3	1:200	Jackson Immunotech, USA
Anti-TIM−3	Goat-anti-rabbit IgG; Cy5	1:200	Jackson Immunotech, USA
Anti-Gal−9	Goat-anti-mouse IgG, Cy3	1:200	Jackson Immunotech, USA

TIM-3 is significantly (p = 0.049) upregulated in acute covid-19 EVTs of male fetuses and non-significantly (p = 0.061) upregulated in male post covid-19 EVTs (Fig. 1**a** control, Fig. 1**b** male covid-19 placenta, Fig. 1**c** male post covid-19). In female placentas, TIM-3 is unchanged (Fig. 1**d, e, f**). A summary of the staining results is presented in Fig. 1**g**.

3.2. Expression of TIM-3 in decidual macrophages

In male decidual macrophages, TIM-3 is unchanged (Fig. 2**a, b, c**). TIM-3 is expressed in decidual macrophages within the decidual stroma of normal control third trimester placentas. There are significant differences in TIM-3 expression in macrophages within decidual tissue between female control (Fig. 2**d**) and female outcome placentas of women with acute Covid-19 infection (Fig. 2**e**, median control percent score 50 versus median percent score of 75 in acute Covid-19 infection placentas; p = 0.021). In female post Covid-19 placentas, there were no differences for TIM-3 expression in decidual macrophages (Fig. 2**f**) compared to control female macrophages. A summary of the staining results is presented in Fig. 2**g**.

3.3. Expression of TIM-3 in fetal Hofbauer cells

TIM-3 is expressed in abundance within villous tissue of normal control male third trimester placentas (Fig. 3**a**). Significant differences were observed in TIM-3 expression in fetal Hofbauer cells between male control (Fig. 3**a**) and male post Covid-19 infection Hofbauer cells (Fig. 3**c**; median control percent score 75 versus median present score of 60 in post Covid-19 infection placentas; p = 0.023). In female placentas, TIM-3 is unchanged (Fig. 3**d, e, f**). A summary of all staining results in Hofbauer cells is presented in Fig. 3**g**.

3.4. Expression of Gal-9 in fetal Hofbauer cells

Gal-9 is expressed in abundance within villous tissue of normal control male third trimester placentas (Fig. 4**a**). Significant differences were observed in Gal-9 expression in fetal Hofbauer cells between male control (Fig. 4**a**) and male Covid-19 infection Hofbauer cells (Fig. 4**b**; median control percent score 20 versus median present score of 50 in covid-19 infection placentas; p = 0.035). Gal-9 is significantly downregulated in post Covid-19 male Hofbauer cells (Fig. 4**c**, p = 0.005) compared to acute covid placentas. In female placentas, Gal-9 follows the same trend as in male placentas but without statistical significance (Fig. 4**d, e, f**). A summary of all staining results in Hofbauer cells is presented in Fig. 4**g**.

3.5. Expression of Gal-9 in decidual macrophages

In male decidual macrophages, Gal-9 is unchanged (Fig. 5**a, b, c**). Gal-9 is expressed in decidual macrophages within the decidual stroma of normal control third trimester placentas. Nearly significant differences were observed in Gal-9 expression in macrophages within decidual tissue between female control (Fig. 5**d**) and female outcome placentas of women with acute Covid-19 infection (Fig. 5**e**, median control percent score 9 versus median percent score of 13 in acute Covid-19 infection placentas; p = 0.053). In female post Covid-19 placentas, there were no differences for Gal-9 expression in decidual macrophages (Fig. 5**f**) compared to control female macrophages. A summary of the staining results is presented in Fig. 5**g.**

3.6. Identification of TIM-3 expressing cells as extravillous trophoblast cells (EVTs) by double immune fluorescence

Within the male control placenta, we see an abundance of TIM-3-positive cells stained in red (Fig. 6**a**). Cytokeratin-7 (lower left corner, CK7, green) was used as EVT specific marker. Single TIM-3 expression is shown in the upper right corner. Double expression of TIM-3 and CK7 identified TIM-3-positive cells as EVT (Fig. 6**a**) in male control placentas. Within the female control placenta, we also see an abundance of TIM-3-positive cells stained in green (Fig. 6**d**). Again, Cytokeratin-7 (lower left corner, CK7, green) was used as EVT specific marker. Single TIM-3 expression is shown in the upper right corner. Double expression of TIM-3 and CK7 identified TIM-3 positive cells as EVT (Fig. 6**d**) in female control placentas. TIM-3 is expressed on acute Covid-19 male (Fig. 6**b**) and female placentas (Fig. 6**e**). Staining explanation is the same as in controls. Also, in post Covid-19 placentas, TIM-3 is expressed on male (Fig. 6**c**) as well as female extravillous trophoblast cells (Fig. 6**f**). The identification and color explanation are the same as in controls.

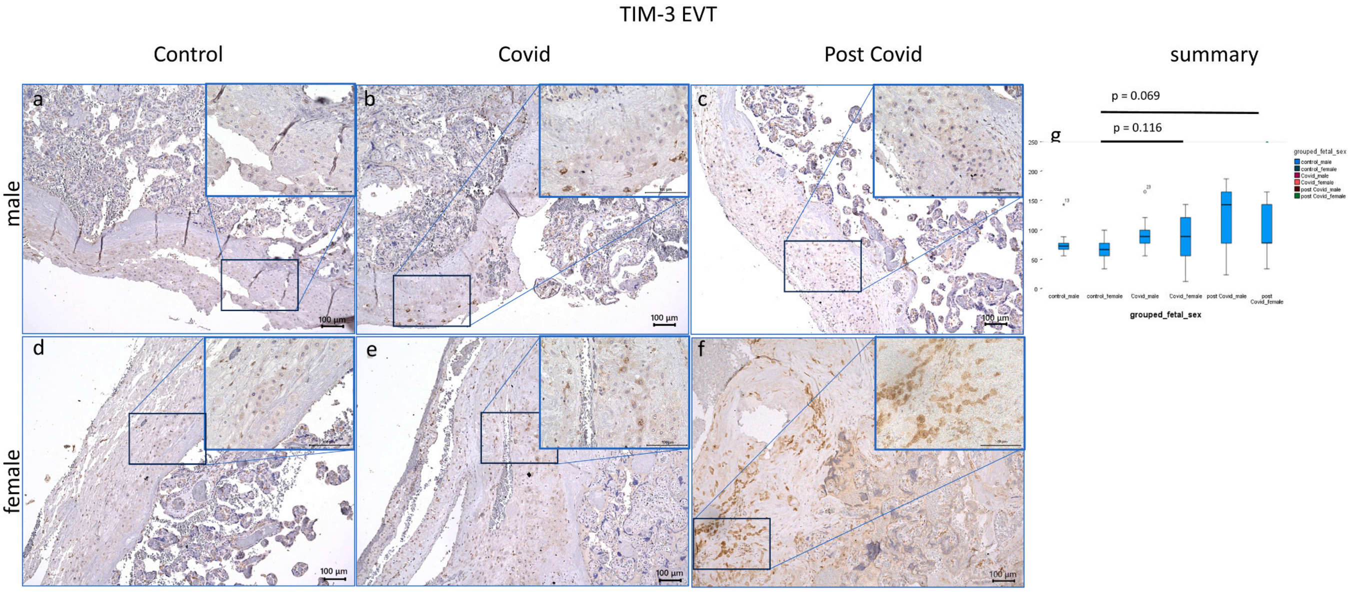

Fig. 1. A low number of TIM-3-positive extravillous trophoblast cells (EVT) can be found at the feto-maternal interphase in healthy control placentas of male fetuses (**a**), magnification 10x and 40x insert. A significantly enhanced expression of TIM-3 (p = 0.049; marked with an asterisk) was observed in acute male covid-19 EVT (**b**), magnification 10x and 40x insert and a nearly significant upregulation of TIM-3 (p = 0.061) was found in post covid EVTs (**c**). In female placentas (**d, e, f**) no significant changes of TIM-3 in EVTs were found. A summary of the staining results is shown as violin plot in g including quartiles and median.

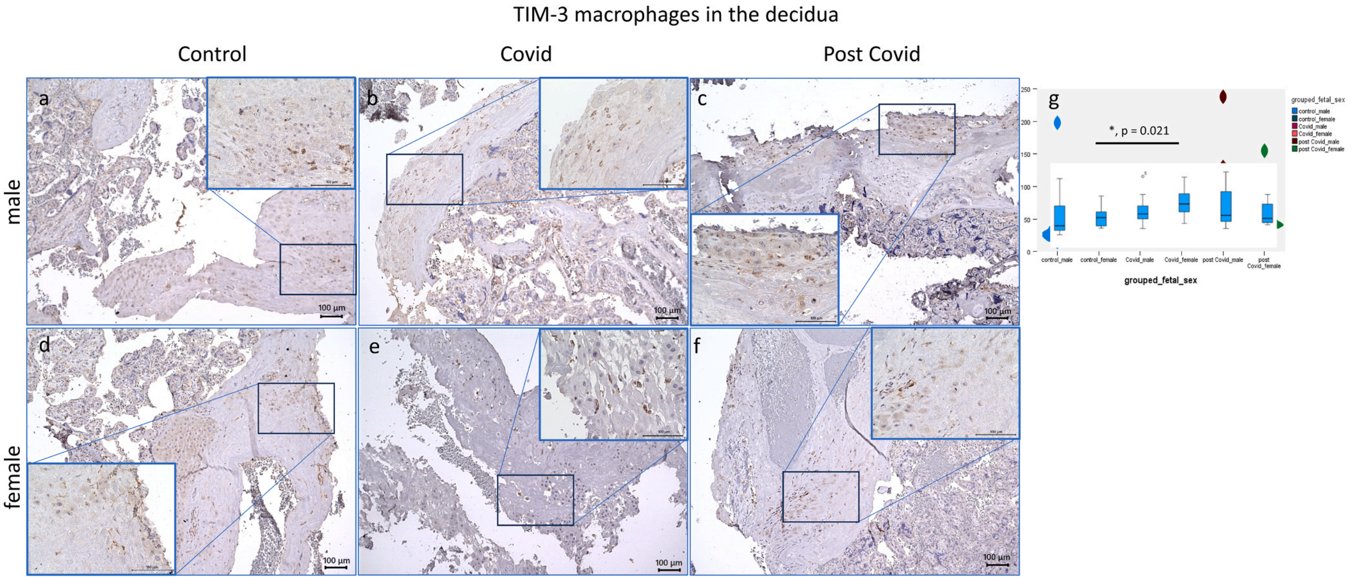

Fig. 2. TIM-3 is expressed in macrophages in low numbers within the feto-maternal interphase in healthy control placentas of male fetuses (**a**), magnification 10x and 40x insert. There was no change in male covid-19 (**b**) and post covid-19 (**c**) decidual macrophages. Significantly enhanced expression of TIM-3 was observed in acute female covid-19 decidual macrophages (**e**) compared to controls (**d**), magnification 10x and 40x insert. There was no significant change in female post covid-19 decidual macrophages in the decidua (**f**). A summary of the staining results is shown as violin plot in **g** including quartiles and median. Significant differences are marked with an asterisk and the p-value is stated.

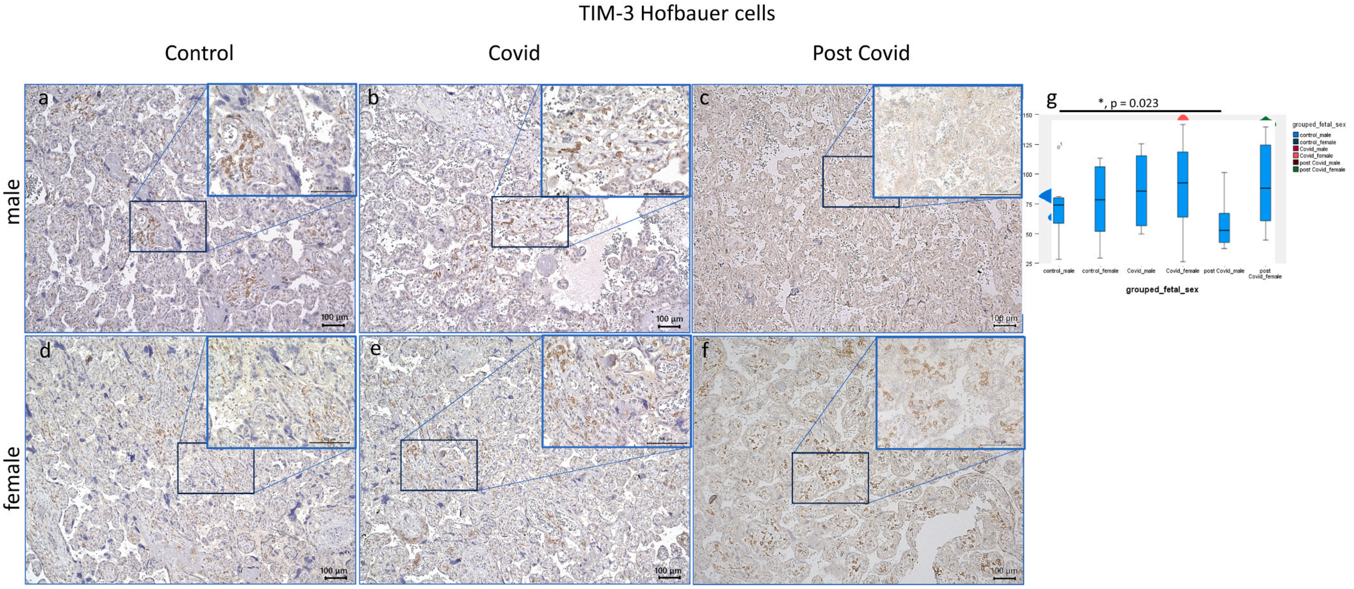

Fig. 3. TIM-3 is expressed in fetal Hofbauer cells in relative high numbers in healthy control placentas of male (**a**) and female (**d**) origine, magnification 10x and 40x. Significantly diminished expression of TIM-3 was observed in post-covid-19 male Hofbauer cells (**c**), whereas in covid-19 male Hofbauer cells (**b**) no significant change could be observed, magnification 10x and insert 40x. There was no significant change in female covid-19 and post-covid-19 Hofbauer cells (**e, f**). A summary of the staining results is shown as violin plot in **g** including quartiles and median. Significant differences are marked with an asterisk and the p-value is stated.

3.7. Identification of TIM-3 expressing cells as decidual macrophages by double immune fluorescence

In acute Covid-19 placentas TIM-3 is expressed on some cells that are not EVTs as seen in Fig. 7**b** (male and **7e** (female). Therefore, we performed double immune fluorescence staining for the identification of these cells within the decidua of the placentas of male (**7a)** and female controls (**7d)**, male acute Covid-19 placentas (**7b)** and female acute Covid-19 placentas (**7e**), male post Covid-19 placentas (**7c)** and female post Covid-19 placentas (**7 f**). TIM-3 positive cells were stained in red (upper right corner in Fig. 7**a, b, c, d, e and f**). Double immunofluorescence with a CD163 antibody (stained in green) and single expression of CD163 in lower left corner in Fig. 7**a, b, c, d, e and f**) showed that TIM-3 positive cells are M2 polarized macrophages.

3.8. Identification of TIM-3 expressing cells in villous tissue by double immune fluorescence

TIM-3 is expressed in abundance within villous tissue of third trimester placentas. TIM-3 positive cells were stained in red (upper right corner in Fig. 8**a, b, c, d, e and f**). Double immunofluorescence with a CD163 antibody (stained in green) and single expression of CD163 in

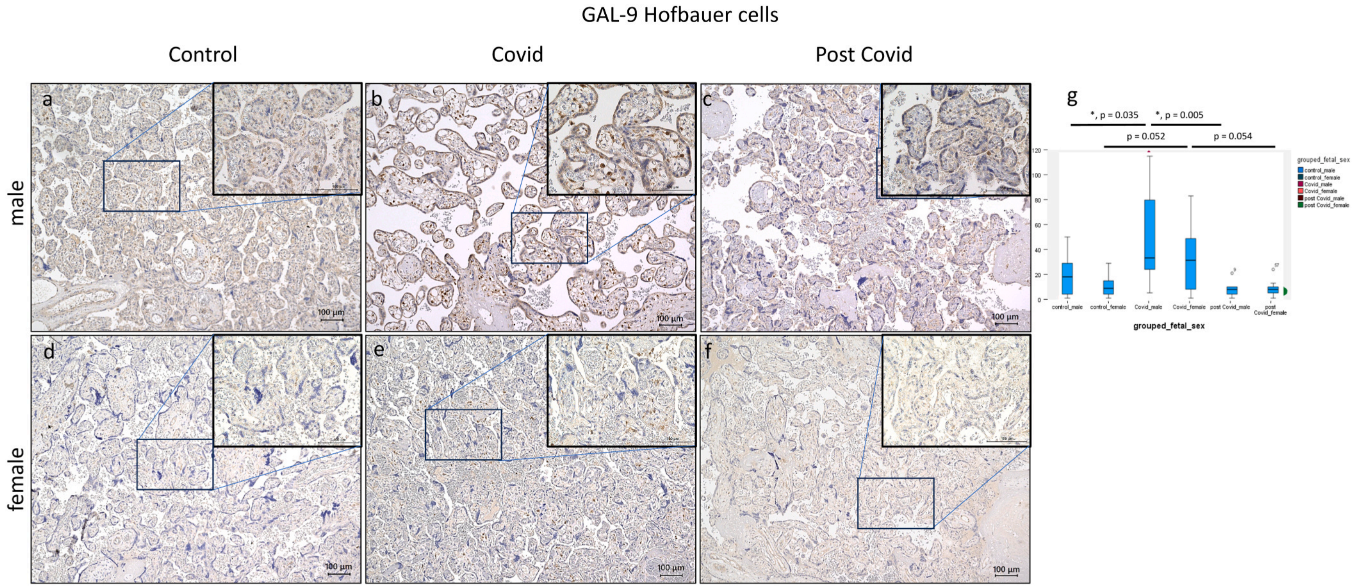

Fig. 4. A low to median expression of gal-9 was identified in Hofbauer cells in healthy control placentas of male fetuses (**a**), magnification 10x and insert 40x. A significant upregulation of gal-9 was observed in male covid-19 Hofbauer cells (**b**) with a significant downregulation in post covid-19 male Hofbauer cells (**c**), magnification 10x and 40x insert. The same trend could be seen in female Hofbauer cells without reaching statistical significance (**d, e, f**). A summary of the staining results is shown as violin plot in **g** including quartiles and median. Significant differences are marked with an asterisk and the p-value is stated.

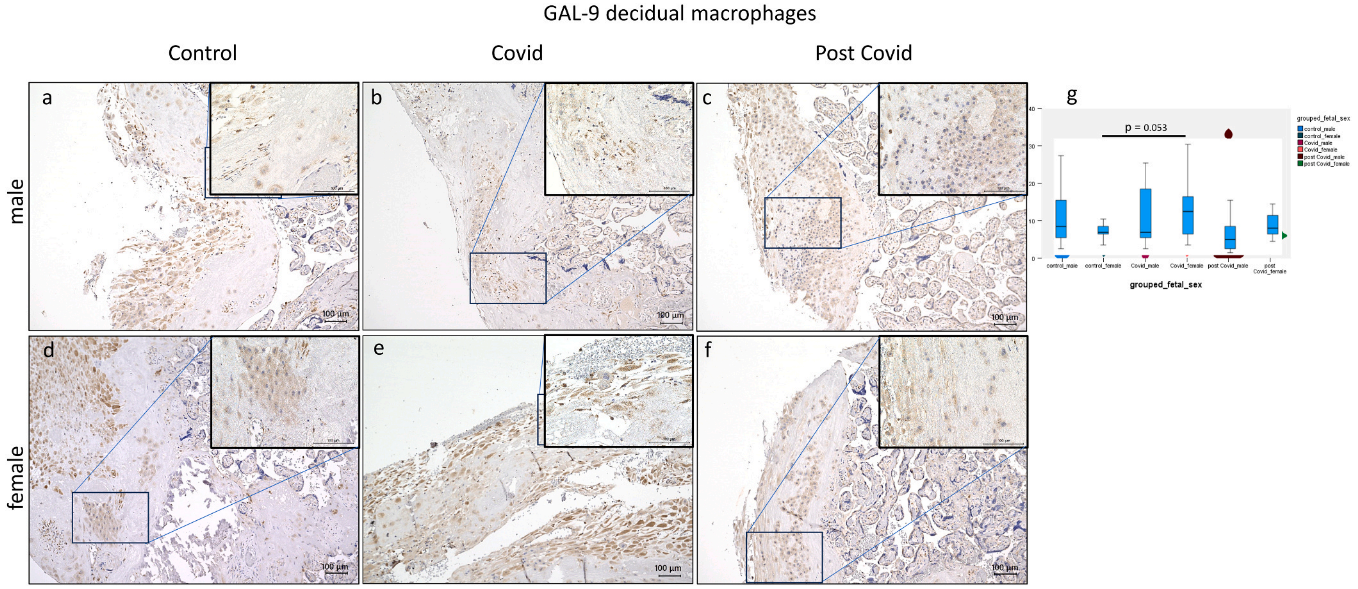

Fig. 5. Gal-9 is expressed in decidual macrophages within the feto-maternal interphase in healthy control placentas of male (**a**) and female fetuses (**d**), magnification 10x and 40x insert. There was no change in male covid-19 (**b**) and post covid-19 (**c**) decidual macrophages. Nearly significantly enhanced expression of gal-9 was observed in acute female covid-19 decidual macrophages (**e**) compared to controls (**d**), magnification 10x and 40x insert. There was no significant change in female post covid-19 decidual macrophages in the decidua (**f**). A summary of the staining results is shown as violin plot in **g** including quartiles and median.

lower left corner in Fig. 8**a, b, c, d, e and f**) showed that TIM-3 positive cells are fetal Hofbauer cells within villous tissue.

3.9. Identification of Gal-9 expressing cells in villous tissue by double immune fluorescence

Gal-9 is expressed in abundance within villous tissue of third trimester placentas. Gal-9 positive cells were stained in red (upper right corner in Fig. 9**a, b, c, d, e and f**). Double immunofluorescence with a CD163 antibody (stained in green) and single expression of CD163 in lower left corner in Fig. 9**a, b, c, d, e and f**) showed that Gal-9 positive cells are fetal Hofbauer cells within villous tissue. Gal-9 expression was found in the cytoplasm as well as in the nucleus of Hofbauer cells. DAPI (blue) together with Gal-9 (red) result in pink staining if Gal-9 locates in the nucleus.

3.10. Identification of Gal-9 expressing cells as decidual macrophages by double immune fluorescence

In acute Covid-19 placentas Gal-9 is expressed on some cells that are not EVTs as seen in Fig. 10**b** (male and 10**e** (female). Therefore, we performed double immune fluorescence staining for the identification of these cells within the decidua of the placentas of male (**10a)** and female controls (**10d)**, male acute Covid-19 placentas (**10b)** and female acute

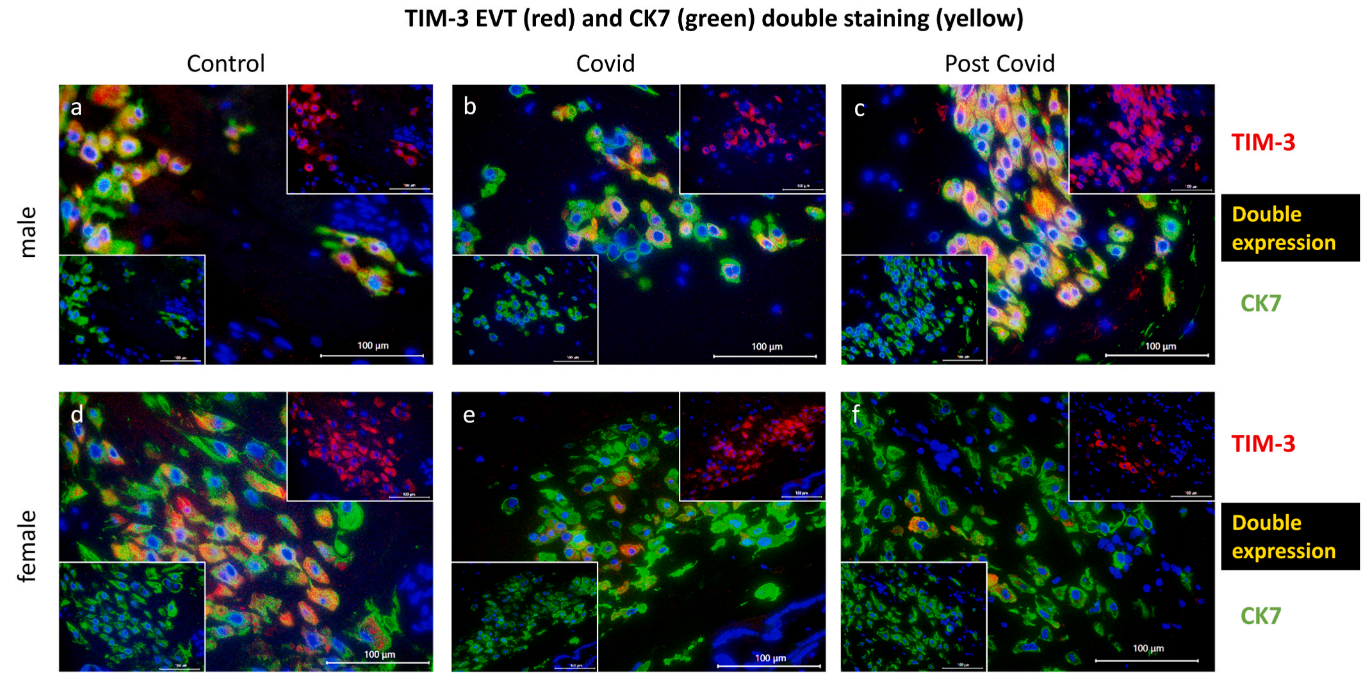

Fig. 6. TIM-3-positive cells (red fluorescence) of male control placentas (**a**) as well of female controls (**d**) are in the majority positive for CK-7 (green fluorescence) and therefore extravillous trophoblasts (EVT), magnification 40x. The same staining pattern was seen in acute covid male (**b**), female placentas (**e**) and in post-covid 19 male (**c**), female placentas (**f**).

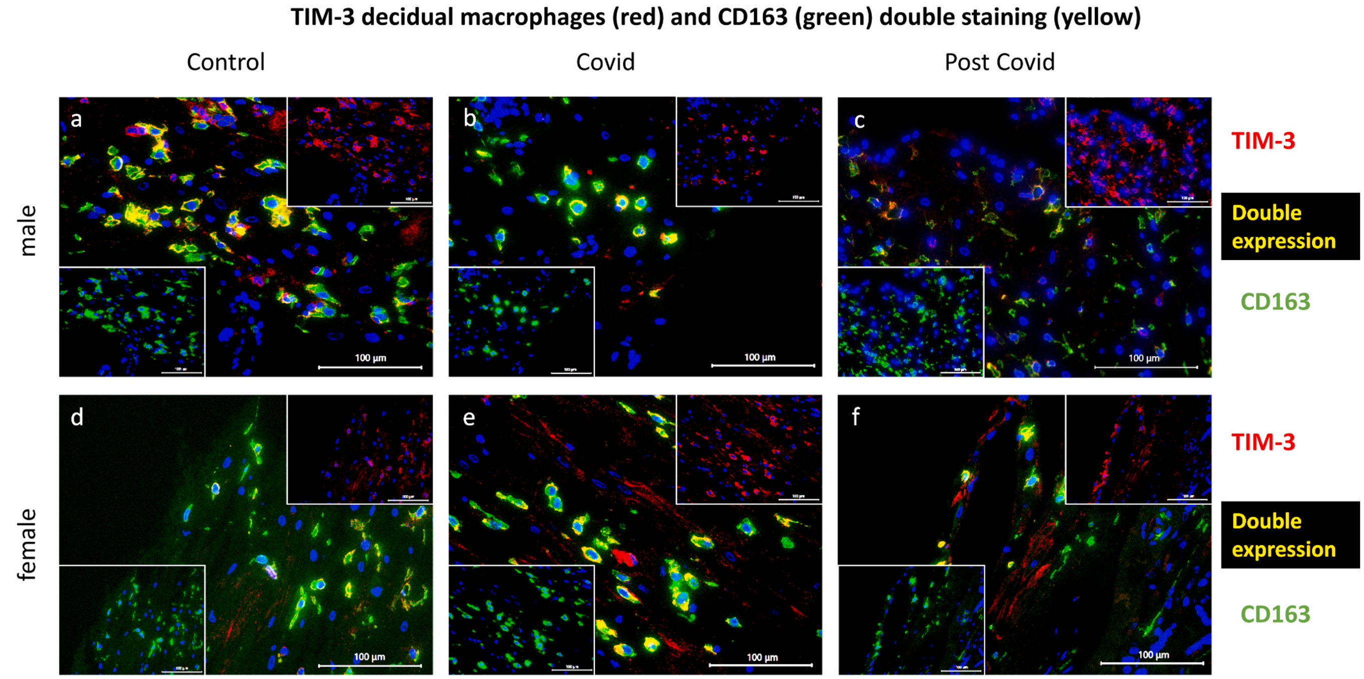

Fig. 7. TIM-3-positive cells (red fluorescence) of male control decidua (**a**) as well of female controls (**d**) are to some content also positive for CD163 (green fluorescence) and therefore decidual macrophages, magnification 40x. The same staining pattern was seen in acute covid male (**b**), female placentas (**e**) and in post-covid 19 male (**c**), female placentas (**f**).

Covid-19 placentas (**10e**), male post Covid-19 placentas (**10c**) and female post Covid-19 placentas (**10 f**). Gal-9 positive cells were stained in red (upper right corner in Fig. 10**a, b, c, d, e and f**). Double immunofluorescence with a CD163 antibody (stained in green) and single expression of CD163 in lower left corner in Fig. 10**a, b, c, d, e and f**) showed that Gal-9-positive cells are M2 polarized macrophages. Gal-9 expression was found in the cytoplasm as well as in the nucleus of decidual macrophages. DAPI (blue) together with Gal-9 (red) result in pink staining if Gal-9 locates in the nucleus.

3.11. Co-expression of CD163, Gal-9 & TIM-3 in villous tissue by triple immune fluorescence

Gal-9 & TIM3 are expressed in abundance within villous tissue of

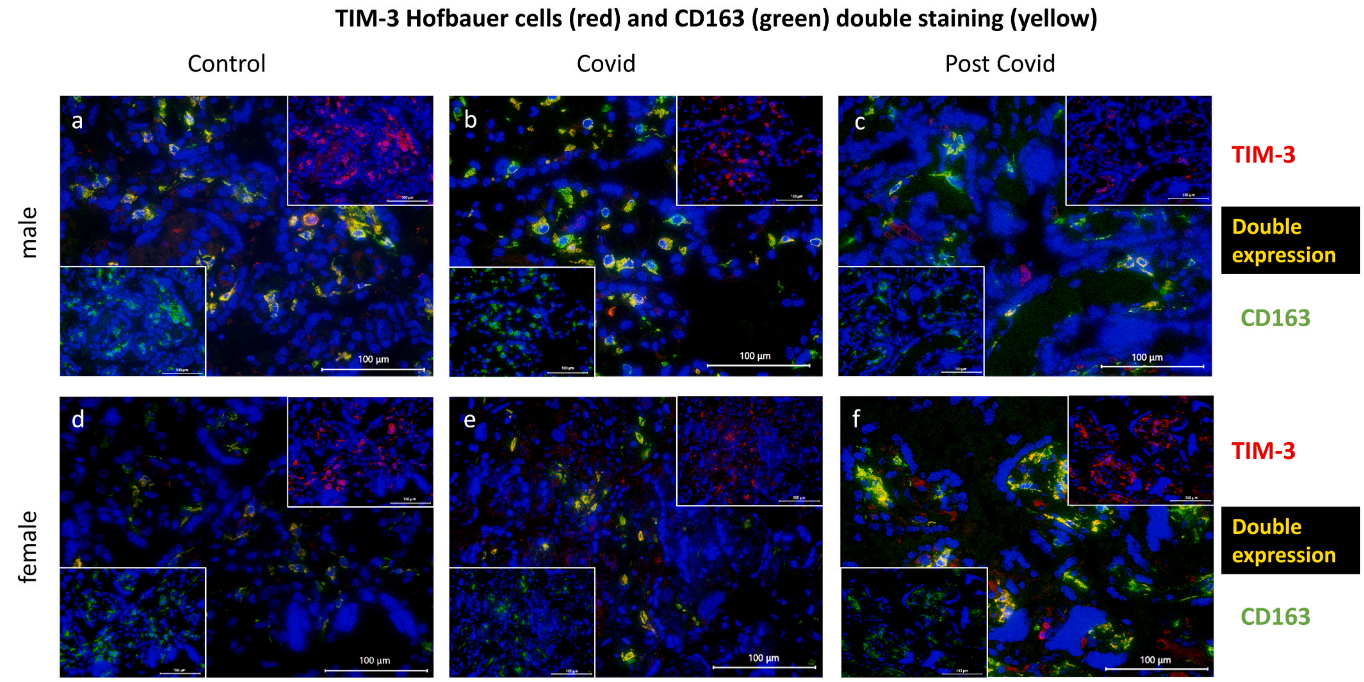

Fig. 8. Within villous tissue, TIM-3-positive cells (red fluorescence) of male control placentas (**a**) as well of female controls (**d**) are also positive for CD163 (green fluorescence) and therefore fetal Hofbauer cells, magnification 40x. The same staining pattern was seen in acute covid male (**b**), female placentas (**e**) but in post-covid 19 male (**c**) Hofbauer cells, TIM-3 staining is diminished but unchanged in female placentas (**f**).

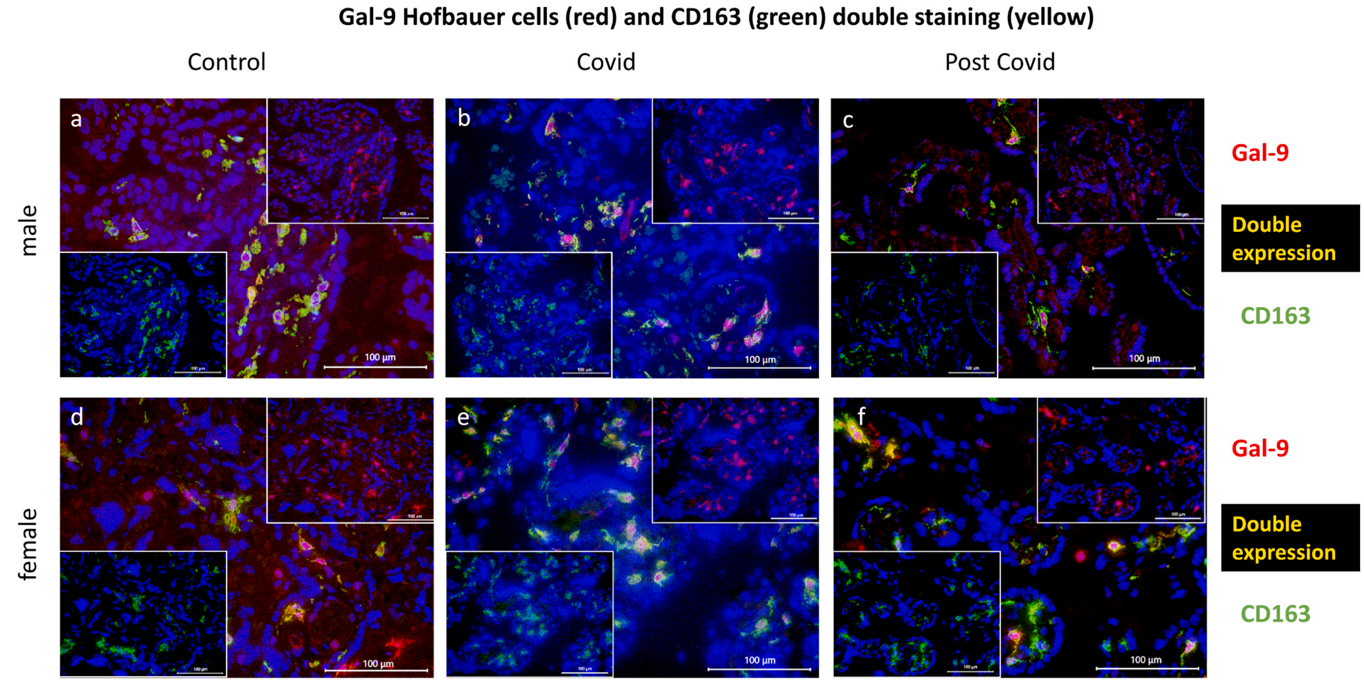

Fig. 9. Within villous tissue, gal-9-positive cells (red fluorescence) of male control placentas (**a**) as well of female controls (**d**) are also positive for CD163 (green fluorescence) and therefore fetal Hofbauer cells, magnification 40x. In acute covid male (**b**) Hofbauer cells, there is a very strong nuclear gal-9 expression whereas in female placentas (**e**) the expression is not changed. In post-covid 19 male (**c**) Hofbauer cells, gal-9 staining is diminished compared to acute covid-19 Hofbauer cells, but unchanged in female placentas (**f**).

third trimester placentas. Gal-9 positive cells were stained in red (upper right corner in Fig. 11a, b, c, d, e and f) and TIM-3 positive cells were stained in pink (upper left corner in Fig. 11a, b, c, d, e and f). Triple immunofluorescence with a CD163 antibody (stained in green) and single expression of CD163 in lower left corner in Fig. 11a, b, c, d, e and f) showed that Gal-9 positive cells are fetal Hofbauer cells within villous tissue.).

3.12. Co-expression of CD163, Gal-9 & TIM-3 in decidual macrophages by triple immune fluorescence

Gal-9 & TIM3 are expressed in decidual macrophages of third

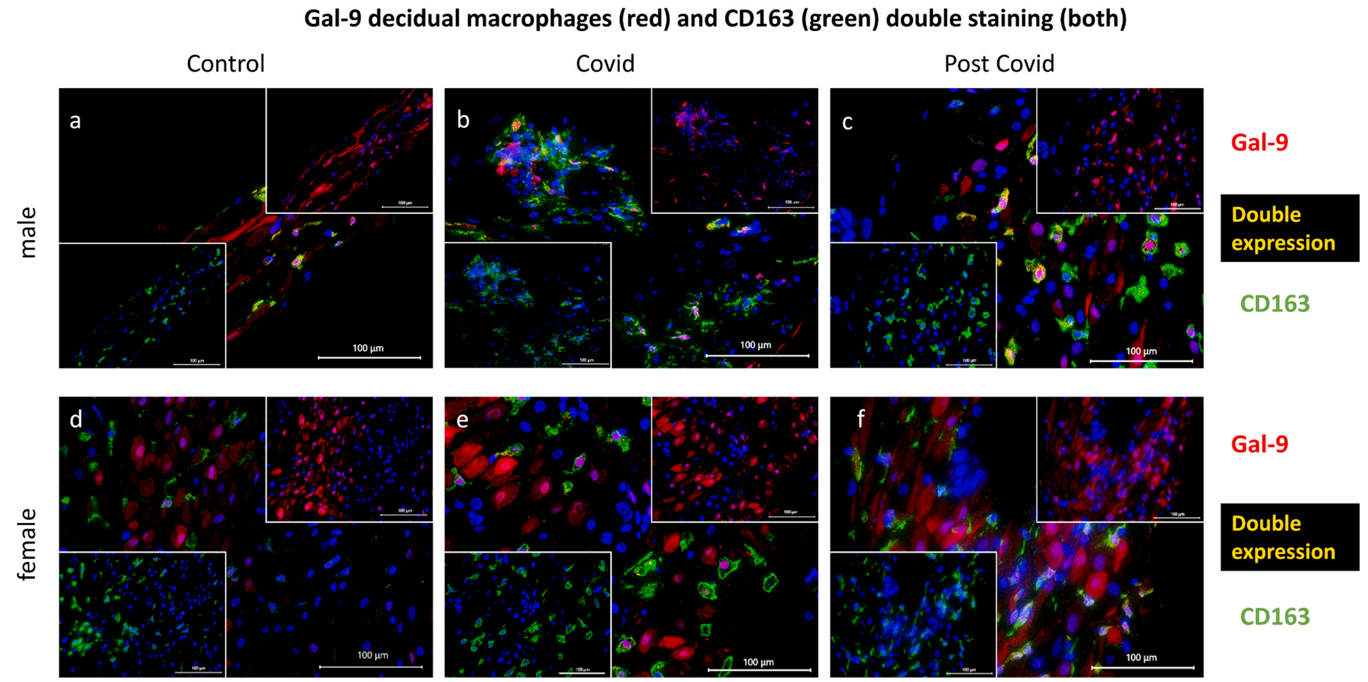

Fig. 10. Gal-9-positive cells (red fluorescence) of male control decidua (**a**) as well of female controls (**d**) are to some content also positive for CD163 (green fluorescence) and therefore decidual macrophages, magnification 40x. In male acute covid-19 decidua's, the number of cells, positive for both, gal-9 and CD163 seems to be higher compared to controls (**b**). The same staining pattern as in controls was seen in female placentas (**e**) and in post-covid 19 male (**c**) and female placentas (**f**).

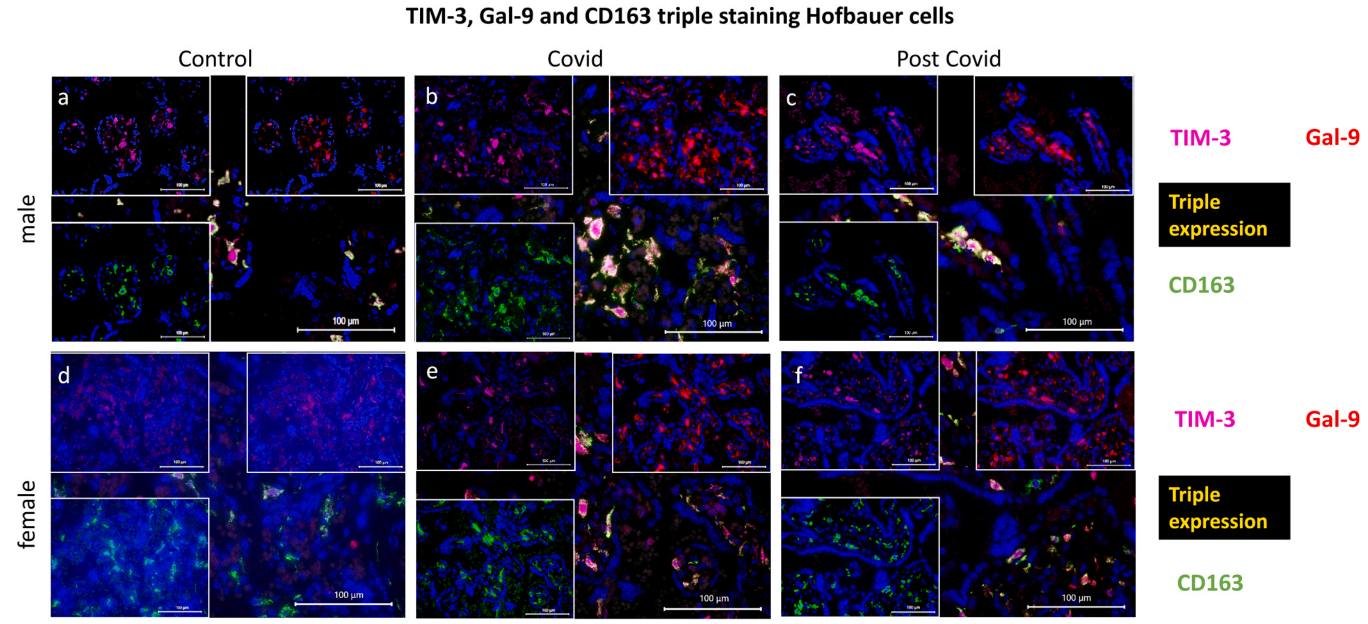

Fig. 11. Gal-9-positive cells (red fluorescence) + TIM-3-positive cells (pink fluorescence) of male control villous tissue cells (a) as well of female controls (d) are positive for CD163 (green fluorescence) and therefore villous Hofbauer cells, magnification 40x. In male acute covid-19 decidua's, the number of cells, positive for both, gal-9 + TIM-3 and CD163 seems to be higher compared to controls (b). The same staining pattern as in controls was seen in female placentas (e) and in post-covid 19 male (c) and female placentas (f).

trimester placentas. Gal-9 positive cells were stained in red (upper right corner in Fig. 12a, b, c, d, e and f) and TIM-3 positive cells were stained in pink (upper left corner in Fig. 12a, b, c, d, e and f). Triple immunofluorescence with a CD163 antibody (stained in green) and single expression of CD163 in lower left corner in Fig. 12a, b, c, d, e and f) showed that Gal-9 positive cells are decidual macrophages.

4. Discussion

Within this study, we could show that TIM-3 is significantly upregulated in male extravillous trophoblast cells in the acute phase of Covid-19. On the other hand, TIM-3 was significantly downregulated in Hofbauer cells from male placentas after Covid-19. Gal-9, the ligand for TIM-3, was significantly upregulated in Hofbauer cells in acute Covid-19

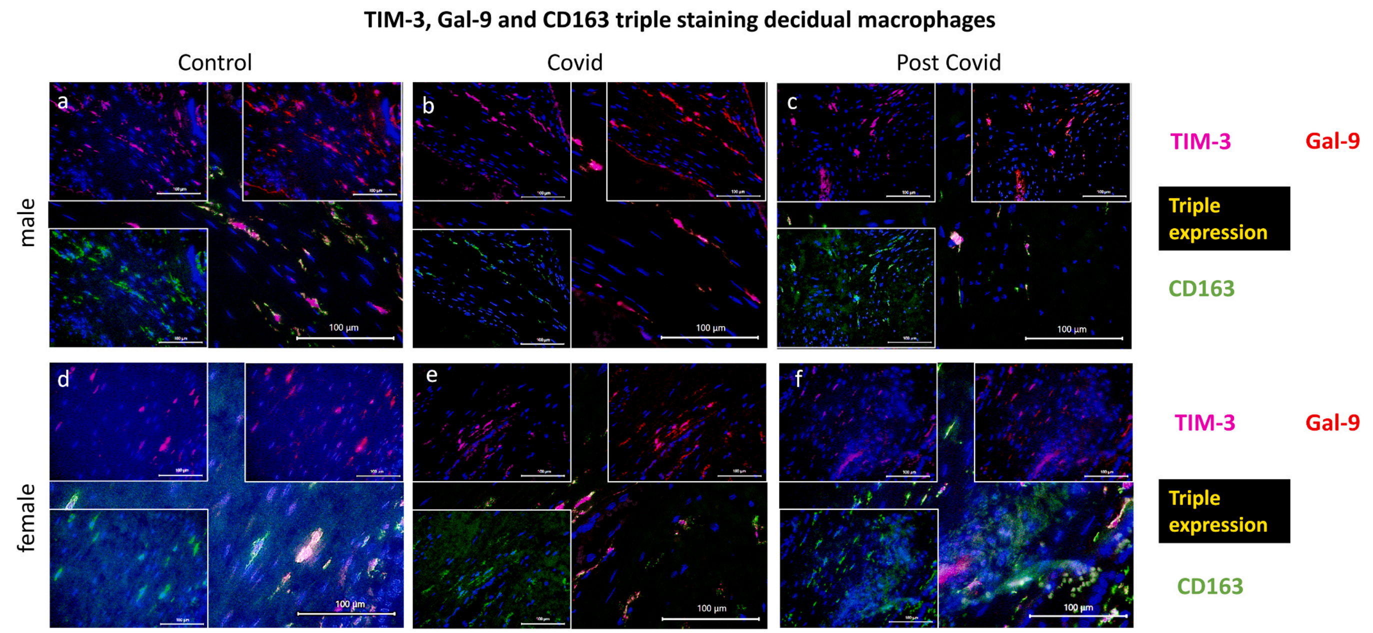

Fig. 12. Gal-9-positive cells (red fluorescence) + TIM-3-positive cells (pink fluorescence) of male control decidua (a) as well of female controls (d) are positive for CD163 (green fluorescence) and therefore decidual macrophages, magnification 40x. In male acute covid-19 decidua's, the number of cells, positive for both, gal-9 + TIM-3 and CD163 seems to be higher compared to controls (b). The same staining pattern as in controls was seen in female placentas (e) and in post-covid 19 male (c) and female placentas (f).

infected male placentas and again downregulated after Covid-19 in male Hofbauer cells. Triple immunofluorescence analysis showed that male Hofbauer cells (analyzed by CD163 staining) were positive for both TIM-3 and Gal-9 in placentas of acute Covid-19 patients. In addition, in female placentas, TIM-3 is significantly upregulated in acute Covid-19 decidual macrophages.

Our study showed differences in TIM-3/Gal-9 expression especially in the post Covid-19 male Hofbauer cell population. TIM-3 has almost completely disappeared from male post Covid-19 Hofbauer cells.

TIM-3 is a key negative immune regulatory molecule that plays a vital role in immunomodulation. TIM-3 can, when abnormally expressed, promote the occurrence and development of autoimmune diseases and tumors and cause immune tolerance (Ma et al., 2023).

A very recent study by Xu et a. showed with combined multiplex immunohistochemical staining, single-cell transcriptomics and spatial transcriptomics data, the spatial distribution and expression of Galectin-9/TIM-3 at the maternal-fetal interface with single-cell resolution (Xu et al., 2025). Xu et al. identified Hofbauer cells as the primary fetal source of Galectin-9, rather than trophoblasts, offering a fresh perspective on the mechanisms underlying immune tolerance at the maternal-fetal interface (Xu et al., 2025).

TIM-3 exerts a significant impact on the antiviral immune response in chronic viral infections (Ma et al., 2023). A former study showed that in a chronic lymphocytic choriomeningitis virus (LCMV) infection model, virus-specific $CD8^+$ T cells expressing TIM-3 showed lower production of proinflammatory cytokines such as IL-2, TNF-α, and IFN-γ (Jin et al., 2010). Some studies showed changes in TIM-3 expression during Covid-19 infection (Paces et al., 2020). During the acute phase of the infection, TIM-3 expression on the surface of immune cells is increased (Barnova et al., 2021). This is in line with our findings, showing an upregulation of TIM-3 in male extravillous trophoblast cells as well as in decidual macrophages of female placentas in acute Covid-19 cases.

Another striking finding was, that we identified a significant downregulation of TIM-3 in male post-Covid-19 Hofbauer cells. This downregulation only in male Hofbauer cells is accompanied with a significant downregulation of the TIM-3 ligand Gal-9 in post Covid-19 compared to acute Covid-19 cases.

A similar result was found in one of our previous studies showing that on Hofbauer cells within the chorionic villi, TIM-3 expression was significantly downregulated in preeclamptic cases but in that case without a sex-specific differences (Mittelberger et al., 2024). When their specific ligand is recognized, they have an inhibitory effect on the immune system. They achieve this by negatively regulating effector cells, such as T-cells. These molecules are central to immune regulation during tumor growth, autoimmunity and infections (Kandel et al., 2021). Reduced TIM-3 expression on Hofbauer cells could lead to inflammation within the chorionic villi due to the reduced binding capacity for Gal-9. Hofbauer cells originate from yolk sac macrophages and migrate to other organs through fetal development (Stremmel et al., 2018). This could cause inflammation and organ damage in later life of the infants.

Since only male Hofbauer cells showed this effect in the case of Covid-19 infection, particular caution is required in the further development of male offspring after Covid-19 infection.

It has been shown that the protein Gal-9 is upregulated in female acute Covid-19 decidual macrophages but is significantly downregulated in male Hofbauer cells after Covid-19 compared to acute Covid-19 cases, while a significant upregulation of Gal-9 is observed in male acute Covid-19 Hofbauer cells compared to controls. The latter effect was only significant in male offspring but not in female offspring. The downregulation of male Hofbauer cells post Covid-19 could lead to local inflammation, disrupting the feto-maternal tolerance crucial for healthy pregnancy (Mittelberger et al., 2024). A reduction of Gal-9 in Hofbauer cells could result in a proinflammatory reaction in other fetal organs, due to Hofbauer cell migration during fetal development (Mittelberger et al., 2024). Because this downregulation of Gal-9 in the post Covid-19 Hofbauer cell population is only significant in male offspring it could contribute to the already known male disadvantage of male premature and low birthweight infants (Mittelberger et al., 2024). Further research is needed to determine whether there are gender differences in morbidity and mortality among infants born after Covid-19 infection. A study by Li et al. showed that the protein Gal-9 plays a crucial role in promoting tube formation during early placental development (Li et al., 2021). For a better understanding McMahon et al.

investigated the transmission potential of SARS-CoV-2 and the consequences of infection on the developing brain using human ACE2 knock-in a mouse model (McMahon et al., 2023). This study showed that viral transmission to the fetal tissues, including the brain, took place at later stages of development. It also proved that infection predominantly affected male fetuses (McMahon et al., 2023).

Some recent studies have already put forward the hypothesis that fetal gender could influence various endpoints such as fetal development or epigenetic or clinical parameters. Even at the onset of pregnancy, there are indications that there are differences in DNA methylation of the placenta between spontaneously conceived pregnancies and pregnancies resulting from IVF. When examining autosomal cytosine-guanine dinucleotides (CpGs), a breakdown by fetal sex showed that these were only significantly differentiated in males (Lemaire et al., 2025). A large-scale, cross-cohort analysis of multiple tissues also detected more than 10,000 cytosine-guanine dinucleotides that showed different DNA methylation depending on fetal sex (Czamara et al., 2024). Another study on sex-specific DNA methylation and gene expression in the placenta also showed that prenatal methylation and its genetic regulation are strongly influenced by the sex of the child (Tekola-Ayele et al., 2025). In addition, fetal and maternal hormones (e.g., testosterone, estrogens, glucocorticoids) vary depending on gender and could produce gender-specific effects through direct and indirect influence on epigenetic enzymes (Cohen et al., 2025).

Therefore, it must be discussed that especially male offspring of women which survived Covid-19 infection may have carried higher risks for a very rare transfer of the infection to the fetus and for immunological disturbances later in life.

5. Conclusion

The data of our study showed a downregulation of TIM-3 in Hofbauer cells from male placentas after Covid-19 infection. Gal-9, the ligand for TIM-3, was significantly upregulated in Hofbauer cells in acute Covid-19 infected male placentas and again downregulated after Covid-19 in male Hofbauer cells. The reduction of TIM-3 and Gal-9 within Hofbauer cells can trigger proinflammation in other fetal organs by way of the migration of macrophages throughout fetal development. This disturbance in fetal programming specifically affects male offspring. Additional studies are required to confirm these effects.

CRediT authorship contribution statement

Peter Altevogt: Validation, Formal analysis, Data curation. **Udo Jeschke:** Writing – original draft, Supervision, Conceptualization. **Marina C. Seefried:** Writing – original draft, Investigation. **Marei Sammar:** Project administration, Investigation, Data curation. **Ines Lurtz:** Methodology, Formal analysis, Data curation. **Johanna Mittelberger:** Resources, Methodology, Data curation. **Nina Ditsch:** Visualization, Software, Data curation. **Birgit Urban:** Resources, Investigation, Formal analysis. **Christina Kuhn:** Resources, Methodology, Investigation. **Christian Dannecker:** Resources, Funding acquisition. **Manuela Franitza:** Software, Resources, Project administration. **Jonas Bubmann:** Writing – review & editing, Visualization, Validation. **Carl Mathis Wild:** Writing – review & editing, Software, Methodology.

Declaration of Competing Interest

N.D. reports funding from MSD, Novartis, Pfizer, Roche, AstraZeneca, TEVA, Mentor, and MCI Healthcare. C.D. is funded by Roche, AstraZeneca, TEVA, Mentor, and MCI Healthcare. U.J. received travel money from pfm medicals. All other authors declare no conflict of interest.

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