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Regulatorische T-Zellen und epigenetische Veränderungen in der Präeklampsie

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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

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München, den 11.06.2023	Laura Hahn
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Abkürzungsverzeichnis

ac	acetylierte Histonmodifikation
BeWo	Trophoblasten-Modell-Zelllinie
BMI	Body-Maß-Index in kg/m^2
CCL22	CC-Chemokin-Ligand 22
CCR4	CC-Chemokinrezeptor Typ 4
CD127	Cluster-of-Differentiation 127; Oberflächenmarker zur Klassifizierung von Zellen; der hochgestellte Zusatz zeigt die Expression von CD127 der untersuchten Zellen an (low/dim = wenig CD127, high = viel CD127, - = kein CD127)
CD25	Cluster-of-Differentiation 25; Oberflächenmarker zur Klassifizierung von Zellen ($CD25^+$ = auf den Zellen exprimiert, $CD25^-$ = auf den Zellen nicht exprimiert)
CD4	Cluster-of-Differentiation 4; Oberflächenmarker zur Klassifizierung von Zellen ($CD4^+$ = auf den Zellen exprimiert, $CD4^-$ = auf den Zellen nicht exprimiert)
CK7	Zytokeratin 7, Marker für Trophoblasten
DNA	Desoxyribonukleinsäure
EOP	Early-Onset-Präeklampsie, Beginn vor 34. SSW
EVT	Extravillöser Trophoblast
FasL	Fas-Ligand; Auslösung von Apoptose durch den Fas-FasL-Weg
FoxP3	forkhead box protein 3
Gal-1	Galektin-1
Gal-2	Galektin-2
Gal-9	Galektin-9
H3K4me	methylierter Lysinrest 4 des Histon 3
$H3K4me^1$	einfach methylierter Lysinrest 4 des Histon 3
$H3K4me^3$	trimethylierter Lysinrest 4 des Histon 3
H3K9	Lysinrest 9 des Histon 3

H3K9ac	acetylierter Lysinrest 9 des Histon 3
H3K27	Lysinrest 27 des Histon 3
H3K27me^3	trimethylierter Lysinrest 27 des Histon 3
H3K27ac	acetylierter Lysinrest 27 des Histon 3
H3K79	Lysinrest 79 des Histon 3
HDAC	Histondeacetylase
HDAC3	Histondeacetylase 3
IUGR	Intrauterine Wachstumsretardierung
MDC	Macrophage derived chemokine
me	methylierte Histonmodifikation
MMP2	Matrix-Metalloproteinase-2
MMP9	Matrix-Metalloproteinase-9
mRNA	messenger Ribonukleinsäure, Boten-RNA
PE	Präeklampsie
PlGF	placental growth factor, plazentarer Wachstumsfaktor
PPARγ	Peroxisom-Proliferator-aktivierter-Rezeptor gamma
RxRα	Retinoid-X-Rezeptor alpha
SSW	Schwangerschaftswoche
T0070907	selektiver PPARγ Antagonist
Th2-Zellen	Typ-2-T-Helferzellen
Treg	regulatorische T-Zelle
TUNEL	TdT-mediated dUTP-biotin nick end labeling
VEGF	vascular endothelial growth factor, Gruppe vaskulärer Wachstumsfaktoren

Abbildungsverzeichnis

Publikationsliste

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Meister S, **Hahn L**, Beyer S, Paul C, Mitter S, Kuhn C, et al. Regulation of Epigenetic Modifications in the Placenta during Preeclampsia: PPARgamma Influences H3K4me3 and H3K9ac in Extravillous Trophoblast Cells. Int J Mol Sci. 2021;22(22).

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2. Conference Paper

Hahn L, Beyer S, Kolben TM, Schmoeckel E, Mayr D, Anz D, et al. Verminderte Rekrutierung regulatorischer T-Zellen bei erhöhter CCL22-Expression in der Präeklampsie. Kongressabstracts zur Tagung 2020 der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe (DGGG)2020.

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Hahn L, Meister S, Mannewitz M, Beyer S, Corradini S, Hasbargen U, et al. Gal-2 führt zu einer Erhöhung von H3K4me3 und H3K9ac in Trophoblasten und in der Präeklampsie. 96 Kongress der Bayerischen Gesellschaft für Geburtshilfe und Frauenheilkunde eV ‚BGGF 2022' Unser Nachwuchs –unsere Zukunft2022.

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Dreyer EM, Meister S, Thomann M, **Hahn L**, Keilmann L, Beyer S, et al. Einfluss der psychischen Belastung durch die COVID-19 Pandemie auf das Auftreten von Wochen- bettdepressionen und die Relevanz individueller Stressbewältigungs-Strategien. Kongressabstracts zur Tagung 2022 der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe (DGGG)2022.

3. Geplante Publikationen

Delius M, Kolben T, Nußbaum C, Bogner-Flatz V, Delius A, **Hahn L**, et al. Changes in the rate of preterm children in the COVID-19 pandemic lockdown period – data from a large tertiary German Perinatal Center.

Die vorliegende Dissertation wurde als kumulative Arbeit eingereicht. Grundlage dieser Arbeit sind die folgenden Publikationen:

1. Meister S, Hahn L, Beyer S, Kuhn C, Jegen M, von Schonfeldt V, et al. Epigenetic modification via H3K4me3 and H3K9ac in human placenta is reduced in preeclampsia. J Reprod Immunol. 2021;145:103287.
2. Meister S, Hahn L, Beyer S, Paul C, Mitter S, Kuhn C, et al. Regulation of Epigenetic Modifications in the Placenta during Preeclampsia: PPARgamma Influences H3K4me3 and H3K9ac in Extravillous Trophoblast Cells. Int J Mol Sci. 2021;22(22).
3. Meister S, Hahn L, Beyer S, Mannewitz M, Perleberg C, Schnell K, et al. Regulatory T Cell Apoptosis during Preeclampsia May Be Prevented by Gal-2. International Journal of Molecular Sciences. 2022;23(3).
4. Hahn L, Meister S, Mannewitz M, Beyer S, Corradini S, Hasbargen U, et al. Gal-2 Increases H3K4me3 and H3K9ac in Trophoblasts and Preeclampsia. Biomolecules. 2022;12(5).

Die Bestätigung der Co-Autoren sind vollständig im beigefügten Promotionsantrag zu finden.

I. Einleitung

1. Präeklampsie – Allgemeines

Mit einer Inzidenz von 2-5 % aller Schwangerschaften in Europa und den USA und bis zu 9 % in Afrika handelt es sich bei der Präeklampsie (PE) um eine der häufigsten Schwangerschaftserkrankungen (5, 6). Sie ist eine der wichtigsten Ursachen für maternale, perinatale und neonatale Morbidität und Mortalität und ist für 10-15 % aller maternalen Todesfälle weltweit verantwortlich (7-9).

Die PE wird laut Leitlinie der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe (5) definiert als „jeder (auch vorbestehend) erhöhte Blutdruck ≥ 140/90 mmHg in der Schwangerschaft mit mindestens einer neu auftretenden Organmanifestation, welche keiner anderen Ursache zugeordnet werden kann“. Betroffene Organe können dabei die Niere (Proteinurie > 300 mg/ 24 h), die Leber, das ZNS oder die Lunge sein (6, 10, 11). Zu häufigen Symptomen zählen Kopfschmerzen, Augenflimmern, Oberbauchschmerzen und Ödeme (5, 12). Am häufigsten treten diese im zweiten und dritten Trimenon auf (13-15). Eine Manifestation vor der 20. Schwangerschaftswoche (SSW) deutet auf präexistente Organschäden hin, weshalb in vielen Definitionen eine Erstmanifestation nach der 20. SSW als Diagnosekriterium gilt (5).

Je nach Zeitpunkt der Erstmanifestation kann die PE in eine Early-Onset (EOP, vor der 34. SSW) und eine Late-Onset-Präeklampsie (LOP, nach der 34. SSW) unterteilt werden (16). Die EOP geht dabei mit schweren Symptomen sowie einer erhöhten Komplikations- und Frühgeburtlichkeitsrate einher (17) und betrifft ca. 1 % aller Schwangerschaften in Mitteleuropa (14).

Es sind bereits verschiedene Risikofaktoren für die Entstehung einer PE bekannt, darunter maternales Alter über 35 Jahre, ein BMI über 25 oder Autoimmunerkrankungen. Die Risikoerhöhung durch andere Schwangerschaftserkrankungen und Plazentapathologien lässt auf die Plazenta als ursächliches Organ schließen. (5, 18, 19)

1.1. Aufbau und Funktion der Plazenta

Bei der Plazenta handelt es sich um ein hoch organisiertes und vaskularisiertes Organ (20). Sie verbindet fetales Gewebe mit der Wand des Uterus und weist tumorähnliche Eigenschaften auf (21). Die Plazenta entsteht ab dem fünften Tag nach Fertilisation mit der Differenzierung von Trophoblasten (14). Dies sind epitheliale Zellen der Plazenta (22), die ein stark proliferatives und invasives Wachstum aufweisen können (23).

Ab dem Zeitpunkt der Implantation proliferieren und fusionieren die Trophoblasten, wodurch der Synzytiotrophoblast entsteht (14, 20, 24). Dieser bildet eine Grenzschicht um das fetale Gewebe und sorgt für die Kontrolle des maternofetalen Transfers (6, 14, 20). Ein Teil der Zytotrophoblasten wandert zudem in den Uterus ein und wird somit zu extravillösen Trophoblasten (EVT), die sich zu Zellsäulen anordnen und anschließend ihre Fähigkeit zur Proliferation verlieren (14). Die auswandernden Trophoblasten wandeln die uterinen Spiralarterien um und begrenzen den maternalen Plasmastrom in der Plazenta (24). Es kommt zu einem reduzierten Sauerstoffzufluss in der Plazenta, was zum Schutz des Embryos vor Teratogenese sowie zu einem schnelleren Wachstum des Fetus führt (14). Zusätzlich schützt der verminderte Plasmafluss den Synzytiotrophoblast vor Kontakt mit maternalen Immunzellen (14).

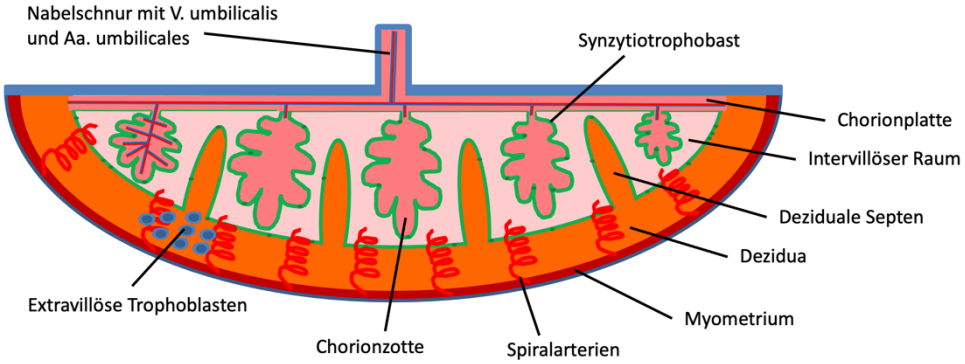

Abbildung 1: Schematischer Aufbau einer Plazenta

Im Laufe der Schwangerschaft übernimmt die Plazenta verschiedene Aufgaben, wie den Nährstofftransport zum Fetus, den maternofetalen Gasaustausch, die fetale Hormonproduktion und ermöglicht eine immunologische Toleranz der Mutter gegenüber des semiallogenen Fetus (14, 20, 25, 26).

1.2. Pathophysiologie der Präeklampsie

Die pathophysiologischen Ursachen der PE sind bislang nicht vollständig geklärt, jedoch ist bekannt, dass eine gestörte Plazentation maßgeblich dazu beiträgt (6, 27). Durch vermehrte Apoptose der EVT (28, 29) und einer dysfunktionalen Immunreaktion in der Plazenta (30, 31) kommt es zu einer gestörten Trophoblasteninvasion in das uterine Gewebe (32), die in einer gestörten Umwandlung der uterinen Spiralarterien resultiert (6, 33-35). Dieses fehlerhafte Remodelling resultiert in einem erhöhten Gefäßwiderstand (36), der Ausschüttung vasokonstriktiver Peptide (6, 37) und in einer daraus folgenden uteroplazentaren Malperfusion (32, 38, 39). Durch diese beiden Faktoren entstehen eine plazentare Insuffizienz und eine chronisch plazentare Ischämie (40). Die entstehende Hypoxie hemmt die Differenzierung der Trophoblasten und führt unter anderem zu lokalen Entzündungsreaktionen (20, 33, 34, 41).

Zudem stellt die lokale Entzündung ein Anzeichen für das Versagen der Anpassung der mütterlichen Immunantwort an den Fetus dar (29). Es scheint in der PE zu einer überschießenden Immunantwort und daraus entstehenden plazentaren und kardiovaskulären Anomalien zu kommen (29, 42).

Eine weitere Theorie zur Entstehung der PE ist die maternale kardiovaskuläre Maladaption (43). Die Schwangerschaft wird dabei als kardiometabolischer Stresstest gedeutet, der eine vorbestehende endotheliale Dysfunktion demaskiert (14). Diese Theorie wird dadurch gestützt, dass chronische Hypertonie und andere kardiovaskuläre Erkrankungen das Risiko einer PE erhöhen (5, 18, 44).

Dies sind jedoch lediglich Theorien und die Pathophysiologie der Präeklampsie ist noch unzureichend geklärt.

1.3. Therapie der Präeklampsie

Eine kurative Therapie zur Behandlung der PE existiert zum aktuellen Zeitpunkt nicht (38). Bei hohem Risiko für die Entwicklung einer PE kann präventiv bis zur 16. SSW Acetylsalicylsäure gegeben werden (5, 14). Zusätzlich können die maternalen Risiken durch eine antihypertensive Therapie mit einem Zielblutdruck von systolisch 130 bis 150 mmHg und diastolisch 80 bis 100 mmHg gesenkt werden (5, 45).

Die einzige Möglichkeit, eine PE tatsächlich zu behandeln, ist die Beendigung der Schwangerschaft und die Entfernung der Plazenta (9, 14, 19, 46). Letztgenannte Maßnahme scheint dabei essentiell zu sein, da Yagel et al. (44) von Fällen berichteten, in denen die Symptome nach Entbindung weiterhin fortbestanden, bis die Plazenta nach 99 Tagen entfernt wurde. Zusätzlich konnten sie zeigen, dass bei dichorioten Zwillingen nach selektivem Fetozid die Symptome binnen 48 Stunden verschwanden (44). Dadurch, dass die Entbindung die einzig mögliche Behandlung darstellt, ist die PE für 40 % der Geburten vor der 35. SSW verantwortlich (47), da nach Diagnosestellung die Schwangerschaft nur zur Vermeidung extremer Frühgeburtlichkeit fortgesetzt werden sollte. Eine Verlängerung der Schwangerschaft über die 37. SSW hinweg ist aufgrund des steigenden maternalen Risikos nicht vertretbar (5). Tritt die PE bereits vor der 24. SSW in Erscheinung, wird aufgrund der Gedeihstörungen des Fetus die Beendigung der Schwangerschaft empfohlen (5).

1.4. Relevanz der Erforschung der Präeklampsie

Wie bereits dargestellt ist weder die Ursache der PE vollständig bekannt, noch existiert eine kurative Therapie. Hierdurch ergibt sich die Möglichkeit eines weiten

Forschungsfeldes. Um mögliche Therapien oder sogar eine Heilung der PE zu finden, ist es zunächst notwendig die Krankheit bestmöglich zu verstehen. Die Erforschung von möglichen ursächlich beteiligten Veränderungen sowie deren Beeinflussbarkeit stellt daher einen der relevantesten Forschungsbereiche der PE dar.

2. Epigenetische Veränderungen in der Präeklampsie

Epigenetik beschreibt die Veränderung der Genexpression ohne eine Veränderung der Nukleotidsequenz der DNA (34, 48-50), welche die Replikation, Transkription sowie den Metabolismus beeinflussen können (25, 49). Zu den epigenetischen Modifikationen zählen unter anderem die DNA-Methylierung, RNA-Interferenz und die Histonmodifikation (20, 51).

Die Präeklampsie zeigt nicht nur während der Schwangerschaft Auswirkungen auf die Gesundheit von Mutter und Fetus, denn es sind bereits weitreichende Langzeitauswirkungen bekannt (34, 52). Bei der Mutter steigt das kardio- und zerebrovaskuläre Risiko nach einer Präeklampsie signifikant an. Außerdem besteht ein erhöhtes Risiko zur Entwicklung eines metabolischen Syndroms, einer Hypertonie und eines Diabetes mellitus Typ 2 (34, 52). Die gesteigerte maternale Langzeitmorbidität ist dabei bis zu 20 Jahre nach der Präeklampsieschwangerschaft nachweisbar (14). Zusätzlich zeigt sich im Erwachsenenalter ein erhöhtes Risiko für die Entwicklung von Karzinomen bei aus Präeklampsieschwangerschaften geborenen Kindern (34). Der Rückschluss, dass diese Langzeitauswirkungen der PE durch epigenetische Veränderungen bedingt sein könnten, entsteht durch die bekannte Beteiligung der Epigenetik an verschiedenen Krankheiten im Erwachsenenalter (34). So trägt die globale Hypomethylierung der DNA im Alter zur Entstehung von Autoimmun- und Krebserkrankungen bei, während die Hypermethylierung von Tumor-Supressor-Genen das Krebsrisiko erhöht. Diese DNA-Methylierungen werden beispielsweise durch das methylierte Histon H3K4 beeinflusst (53).

Es sind bereits unterschiedliche epigenetische Veränderungen in Präeklampsie-Plazenten bekannt, weshalb die Untersuchung dieser Veränderungen von besonderer Bedeutung ist. So sind DNA-Methylierungen mit fehlerhafter Plazentation, erhöhtem Blutdruck und verminderter Trophoblasteninvasion assoziiert (48) und wurden in der PE bereits von zahlreichen Studien nachgewiesen (54, 55). Dabei zeigt sich beispielsweise insbesondere in der EOP eine Hypermethylierung der DNA (20, 56). Auch andere epigenetische Veränderungen - wie die Deregulation der Histone H3K9 und H3K27 - scheinen mit der in Verbindung zu stehen (20).

2.1. Bedeutung der Histonmodifikation in der Präeklampsie

Histone sind Kernproteine, welche die DNA kondensieren und damit die Zugänglichkeit der Gene zur Genexpression kontrollieren (40, 48). Hierzu bildet sich ein Oktamer aus Histonen, um das sich die DNA wickelt (40, 57, 58). Die Histonmodifikation ist eine reversible, posttranslationale Modifikation, die durch Methylierung, Acetylierung oder Phosphorylierung entstehen kann (48, 57, 58). Sie reguliert die Genexpression durch Modulierung des Engegrades der Chromatinverpackung (20, 59) und somit die Zugänglichkeit der DNA (20, 58). Die Histonmodifikation kann sowohl transkriptionsfördernd, als auch -hemmend wirken (48, 50, 58). Die untersuchten Histonmodifikationen trimethyliertes H3K4 und acetyliertes H3K9 sind beide an einem Lysin des Histons H3 modifiziert (20, 57) und wirken transkriptionsfördernd (48, 60).

Die bisherige Forschung zu Histonmodifikationen in der Präeklampsie weist insbesondere auf Veränderungen hin, die zu einer verminderten Zugänglichkeit zur Genexpression führen (58, 59). So finden sich im Tier- sowie im Zellmodell unter Präeklampsiebedingungen verschiedene acetylierte Histonmodifikationen – unter anderem H3K9ac – vermindert (40). Des Weiteren wurde bei PE eine Reduktion des trimethylierten Histons H3K4me^3 in, aus der Nabelschnur isolierten, endothelialen Vorläuferzellen nachgewiesen (52). Weitere Hinweise auf die Beteiligung von Histonmodifikationen an der PE zeigen sich zudem in der veränderten Histonacetylierung in Trophoblasten durch Hypoxie und chronische Ischämie (40, 48). Zusätzlich konnte auch in verschiedenen, an der PE beteiligten Wachstumsfaktoren – beispielsweise beim in der PE verminderten VEGF (vascular endothelial growth factor) – eine dysregulierte Histonmodifikation nachgewiesen werden (55). Es finden sich auch Hinweise darauf, dass die Verminderung von PlGF (placental growth factor) in der PE – das die plazentare Gefäßbildung stimuliert (14) – durch veränderte Histonacetylierung in der Promoterregion verursacht wird (44, 61).

2.2. Mögliche Einflussfaktoren auf die Histonmodifikation

Um die veränderten Histonmodifikationen in der PE bestmöglich interpretieren zu können, ist es sinnvoll, neben der Untersuchung der bloßen Veränderung der Histonmodifikation auch deren Beeinflussbarkeit zu untersuchen.

2.2.1. PPARγ und RxRα – ein transkriptionsförderndes Heterodimer

Der nukleäre Hormonrezeptor PPARγ ist ein ligandenabhängiger Transkriptionsfaktor (62). Um seine transkriptionsfördernde Wirkung zu entfalten, bildet er ein

Heterodimer mit RxRα (retinoic acid X receptor α) (37) und bindet an die DNA (24, 62). Eine Aktivierung von PPARγ führt zu einer erhöhten Rate an Heterodimerisierung mit RxRα (63). Die Verbindung von PPARγ und RxRα zeigt sich auch in der gleichgerichteten Veränderung der Expression nach Zugabe von PPARγ-Agonisten und Antagonisten (64, 65).

PPARγ wurde bisher hauptsächlich im Rahmen der Adipozytedifferenzierung und des Lipidmetabolismus erforscht (24, 46, 66-69), zeigt jedoch auch Einfluss auf die Energiehomöostase und Angiogenese (37, 68, 70). Zusätzlich ist PPARγ an der Karzinogenese – durch Beeinflussung von Zellproliferation, -differenzierung und Apoptose (62, 69, 71) – sowie der Immuntoleranz (62, 69, 72) beteiligt.

In der Plazenta wird PPARγ von endometrialen Stromazellen und Trophoblasten produziert (22, 24, 37, 73), dabei steigt die Expression von PPARγ im Verlauf der Schwangerschaft (74). Es fördert Trophoblastenwachstum, -differenzierung (75) und -invasion (67, 71). Als Heterodimer mit RxRα ist PPARγ an der Entwicklung der Plazenta (22, 67, 71), der Differenzierung des Synzytiums (24), sowie der plazentaren Gefäßentwicklung (37, 62) beteiligt. Zusätzlich ist PPARγ ein an der Implantation des Embryos und Plazentation beteiligtes Protein (46). Es fördert die maternale Immuntoleranz (62, 69, 71, 72) und bietet Schutz gegen Hypoxie und Nährstoffmangel bei insuffizienter Plazentaentwicklung (67). Die Verminderung von PPARγ geht einher mit der Erhöhung proinflammatorischer Zytokine (37, 67, 74) und ist ebenso wie PPARγ-Mutationen (75) mit einem erhöhtem Blutdruck assoziiert (36, 75). Auf mRNA-Ebene wurde bereits eine Verminderung von PPARγ in der PE entdeckt (24), welche bereits Wochen bis Monate vor der Diagnosestellung auftritt (37).

Weitere Hinweise auf die Beteiligung von PPARγ an der Pathophysiologie der PE zeigen sich im Tierversuch. Während bei homozygot fehlendem PPARγ schwere Plazentadefekte mit letalem Ausgang beobachtet werden konnten (74, 75), führte die Inhibition von PPARγ zu einem reduzierten fetalen Wachstum (75) sowie präeklampsieähnlichen Symptomen (22). Zudem scheint eine Aktivierung von PPARγ in vorangeschrittener Schwangerschaft vor intrauterinen Wachstumsretardierungen zu schützen (76). PPARγ stellt somit einen vielversprechenden therapeutischen Ansatzpunkt dar (22, 62).

Die Beziehung von PPARγ und verschiedenen Histonmodifikationen ist vor allem in der Adipogenese ein weit erforschtes Gebiet. Obwohl sich ein Großteil der bisherigen Forschungsergebnisse mit dem Einfluss von Histonmodifikationen auf PPARγ beschäftigt, weisen andere Studien auch auf einen Einfluss von PPARγ auf die

Histonmodifikationen hin. PPARγ zeigt sich in der späten Adipozytendifferenzierung durch H3K4me^3 und H3K9ac positiv beeinflussbar (66, 77-81). Zudem fand sich eine Beeinflussbarkeit der PPARγ-Expression durch Histon modifizierende Proteine (82). Dieser Zusammenhang von Histonmodifikationen und PPARγ fand sich dabei auch bei Autoimmunerkrankungen (83). Dass auch PPARγ einen Einfluss auf die Histonmodifikation haben kann, deuten dagegen die Experimente von Choi et al. (84) an. Zwar fanden auch sie einen hemmenden Effekt der Histondemethylase auf die Adipogenese, jedoch konnten diese Effekte durch Zugabe von exogenem PPARγ rückgängig gemacht werden.

2.2.2. Galektin-2

Galektine gehören zu den Proteinen der Lektin-Familie, binden an β-Galaktosid-Einheiten (85-90) und werden intra- und extrazellulär exprimiert (91). Galektine sind bei verschiedenen Pathologien wie Entzündungen und Infektionen hochreguliert (89) und kommen sowohl in Trophoblasten (85, 86), als auch Trophoblasten-Modell-Zelllinien vor (85). Das untersuchte Galektin-2 (Gal-2) gilt als Stimulator des vaskulären Wachstums und Regulator des Metabolismus (87, 92). Es fördert die Trophoblasteninvasion (87) und ist an der Bildung der Plazenta beteiligt (93). Gal-2 wird bekanntermaßen bei PE, sowohl im peripheren mütterlichen Blut als auch in der Plazenta vermindert exprimiert (87, 92-94).

Der mögliche Zusammenhang von Gal-2 und verschiedenen Histonmodifikationen ist bisher weitgehend unerforscht, jedoch bestehen für andere Galektine Anhalte dafür bei unterschiedlichen Erkrankungen (95-98). Durch die strukturelle und funktionelle Ähnlichkeit der beiden Galektine, lässt insbesondere die Betrachtung von Zusammenhängen zwischen Gal-1 und Histonmodifikationen Rückschlüsse auf Gal-2 zu (89, 99). So führt beispielsweise bei der spinozerebullären Ataxie die Störung einer Histon-Acetyltransferase zur Verminderung von H3K9ac und Gal-1 (95).

Neben bloßen Zusammenhängen finden sich auch Hinweise auf gerichtete Einflüsse zwischen Gal-1 und den Histonmodifikationen. So konnte eine Induktion der Expression von Gal-1 durch HDAC-Inhibitoren (100-103), sowie eine Herunterregulation des Rezeptors von Gal-1 durch Histondeacetylierung nachgewiesen werden (104). Fan et al. (105) zeigten zudem eine verringerte Phosphorylierung und Translokation der Histon-Deacetylase-4 und somit eine verringerte Gentranskription durch Gal-1. Dagegen zeigten Maier et al. (103) eine gewebeselektive Hemmung von Histondeacetylasen in Gliomzellen bei Überexpression von Gal-1.

3. Bedeutung regulatorischer T-Zellen

Regulatorische T-Zellen (Treg) sind eine Subpopulation der $CD4^+$-T-Zellen (106), die 4-8 % der humanen $CD4^+$-T-Zellen im peripheren Blut ausmachen (107) und sich durch die Oberflächenmarker $CD4^+CD25^+CD127^{low}$ und FoxP3 (forkhead box protein) auszeichnen (107-109). FoxP3 ist ein spezifischer Treg-Entwicklungs-Marker (13, 16, 109) und insbesondere in aktivierten Treg hochgradig exprimiert (13). FoxP3 stellt zudem einen wichtigen Transkriptionsfaktor der Treg dar (110) und ist an der Plazentation und plazentaren Angiogenese beteiligt (111).

Treg tragen zur Entwicklung einer Selbsttoleranz (106, 110, 112) bei und spielen eine wichtige Rolle in der Immunhomöostase (110, 112). Zudem wurde bereits eine verminderte Anzahl (112) und gestörte Funktion (113) von Treg im Zusammenhang mit verschiedenen Autoimmunerkrankungen nachgewiesen.

3.1. Rolle der Treg in der Schwangerschaft

Die Schwangerschaft stellt eine hohe Anforderung an das maternale Immunsystem dar, das einerseits die Mutter vor Infektionen schützen (47) und andererseits eine Abstoßungsreaktion gegen den semi-allogenen Fetus verhindern muss (106, 110, 114). Eine zentrale Rolle in der Unterdrückung der Immunantwort (115) und der Erhöhung der Selbsttoleranz (110) spielen Treg durch die Regulation anderer Immunzellen (106), wie die Unterdrückung zytotoxischer T-Zellen (109, 110, 116, 117). Durch die Erhöhung der Toleranz gegenüber des Fetus und die Kontrolle der Implantation und plazentaren Entwicklung (29) sind Treg maßgeblich am Erhalt der Schwangerschaft beteiligt (29, 47, 118, 119). Sobald die Schwangerschaft etabliert ist und das mütterliche Blut in Kontakt mit dem Synzytium tritt, kommt es zu einer zweiten Welle Alloantigener-Exposition (29). Dadurch begründet sich, dass die Anzahl an Treg bis zum zweiten Trimester ansteigt und am Ende des zweiten Trimesters ihren Höhepunkt erreicht (47, 110, 119). Im dritten Trimester fällt die Anzahl an Treg anschließend wieder ab, um die Geburt zu ermöglichen (47, 119).

Eine verminderte Anzahl an Treg kann zum Abort führen (47, 110) und konnte zudem zusammen mit einer Funktionsstörung vorhandener Treg in der PE nachgewiesen werden (29, 110, 111, 119).

3.2. Rekrutierung der Treg durch CCL22

Die Rekrutierung von Treg erfolgt unter anderem durch das Chemokin CCL22 (macrophage-derived-cheomikine, MDC, genannt) (107, 109, 120, 121). CCL22

bindet an den Oberflächenrezeptor CCR4 und induziert dessen Internalisierung (122). Weiterhin trägt CCL22 zur Leukozyten-Homöostase bei und initiiert eine Immunzellaktivierung sowie die antiinflammatorische Th2-Immunantwort (107). Wie der Name MDC andeutet, wird CCL22 von Makrophagen und dendritischen Zellen produziert (115, 123, 124). Zusätzlich findet sich eine Expression in tumorumgebendem Gewebe (121, 125) und an der fetomaternalen Grenzzone (107, 119). Dabei tritt CCL22 jedoch nur in maternalen Stromazellen und Trophoblasten auf, nicht jedoch in fetalen Zellen (107). Eine Induktion der Expression von CCL22 scheint durch ein proinflammatorisches Milieu (115, 125) und Infektionen (126) stattzufinden.

3.3. Zusammenhang von Gal-2 und verschiedenen T-Zellen

Galektine entfalten ihre Wirkung über Transmembransignale durch Galektin-vermittelte Vernetzung von Zelloberflächen-Glykokonjugaten und agieren vorwiegend im Bereich der Proliferation, Zellmigration und Apoptoseinduktion (127). Sie werden dabei unter anderem von Makrophagen, dendritischen Zellen und Treg exprimiert (89). Gal-2 wird – wie unter 2.2.2 beschrieben – in der Plazenta von Präeklampsiepatientinnen vermindert exprimiert (87). Es wirkt immunmodulatorisch (92), induziert die Apoptose aktivierter T-Zellen (87-89, 128, 129) und begünstigt die Differenzierung von Makrophagen (87). Zusätzlich vermindert Gal-2 die Freisetzung proinflammatorischer Zytokine (129).

Über den Zusammenhang von Gal-2 und Treg ist bisher nur wenig bekannt. Loser et al. (89) konnten jedoch bereits nachweisen, dass eine systemische Behandlung mit Gal-2 keinen Einfluss auf die Anzahl oder Funktion von Treg hat.

Anders verhält es sich mit Erkenntnissen zu dem strukturell eng verwandten Gal-1, das 43 % seiner Aminosäurensequenz mit Gal-2 teilt (89, 99). Gal-1 fördert die Induktion, Differenzierung und Expansion von Treg (127, 130) und unterstützt ähnlich wie Gal-2 die Akzeptanz des Fetus durch die Inhibition proinflammatorischer Zytokine und die Förderung der Apoptose aktivierter T-Zellen (89, 127, 128) Dabei führt Gal-2 jedoch zu einer stärkeren Induktion der Apoptose aktivierter T-Zellen als Gal-1 (99). Durch die strukturelle Ähnlichkeit der beiden Galektine, sowie ihre ähnliche Wirkung bezüglich anderer Immunzellen, ist ein ebenfalls positiver Einfluss von Gal-2 auf die Treg zu vermuten.

4. Zielsetzung und Eigenanteil der Veröffentlichungen

4.1. Zielsetzung

Präeklampsie ist eine schwere Schwangerschaftserkrankung mit nicht vollständig geklärter Pathophysiologie und bisher ohne kurative Therapie. Um die Forschung an Therapieoptionen zu ermöglichen ist es zuerst nötig, die Ursache der Erkrankung bestmöglich zu verstehen. Für das Forschungsprojekt wurden zwei bekannte Teilaspekte der PE ausgewählt: die Epigenetik und die fehlerhafte Immunadaptation.

Zielsetzung der Arbeit war es, die Expression ausgewählter Proteine (H3K4me^3, H3K9ac, FoxP3 als Treg-Marker und CCL22) in der Plazenta von Präeklampsiepatientinnen – im Vergleich zu gesunden Kontrollplazenten – anhand von immunhistochemischer Färbungen sowie Immunfluoreszenz-Doppelfärbungen zu untersuchen. Ziel war es dabei jedoch nicht, nur die bloßen Veränderungen darzustellen, sondern auch mögliche Zusammenhänge und Einflussfaktoren anhand von Zellkulturexperimenten zu beurteilen.

Die Ergebnisse dieser Arbeit könnten in weiterführenden Studien nicht nur zu einem besseren Verständnis der pathophysiologischen Grundlagen der Präeklampsie, sondern auch zur Entwicklung von Therapieoptionen beitragen.

4.2. Eigenanteil an den verwendeten Publikationen

Nachfolgend ist der jeweilige Eigenanteil aufgeführt, der zu den Publikationen beigetragen wurde, die dieser kumulativen Dissertation zu Grunde liegen.

Die Beiträge aller weiteren Autoren sind entsprechend der CrediT Taxonomie am Ende der Veröffentlichung aufgelistet und dieser zu entnehmen.

4.2.1. Beitrag zu „Epigenetic modification via H3K4me^3 and H3K9ac in human placenta is reduced in preeclampsia“

Als Beitrag an dieser Originalpublikation fiel mir die immunhistochemische Färbung der Histonmodifikationen H3K4me^3 und H3K9ac zu. Dabei war meine Aufgabe die Durchführung und Auswertung der Färbung sowie die statistische Analyse der Ergebnisse. Weiterhin bestand meine Aufgabe darin, alle Abbildungen für die Publikation zu erstellen und den Revision-Prozess zu unterstützen.

4.2.2. Beitrag zu “The expression of PPARγ and its activity in preeclampsia regulates H3K4me^3 and H3K9ac indicating a defective trophoblast invasion”

Der an dieser Forschungsarbeit geleistete Eigenanteil bestand in der Durchführung immunhistochemischer Färbungen von PPARγ und RxRα, der Immunfluoreszenz-Doppelfärbungen von PPARγ und RxRα mit CK7 bzw. den Histonmodifikationen H3K4me^3/H3K9ac. Des Weiteren führte ich das Zellkulturexperiment mit den HVT-Zellen durch. Zudem wertete ich die Experimente aus und führte die statistische Analyse, sowie deren Interpretation durch. Außerdem war ich für die Datenpflege zuständig.

4.2.3. Beitrag zu “Regulatory T Cell Apoptosis during Preeclampsia May Be Prevented by Gal-2”

Der Beitrag zu diesem Paper bestand in der Durchführung und Auswertung der Experimente. Zudem war ich an der Versuchsplanung der Treg-Apoptose-Färbung sowie des Zellkulturexperiments der Treg beteiligt. Zusätzlich führte ich die statistische Auswertung sowie die Visualisierung der Daten – sowohl in Grafiken als auch Zellbildern – durch. Zusammen mit Dr. Sarah Meister erstellte ich die ursprüngliche Version des Manuskripts. Durch die Zusammenschau der Beiträge zu diesem Paper fiel mir eine geteilte Erstautorenschaft zu. Dr. Sarah Meister war als geteilte Erstautorin zudem an der Projekt- und Versuchsplanung beteiligt und war mit der Datenpflege beauftragt.

4.2.4. Beitrag zu „Gal-2 leads to increased H3K4me^3 and H3K9ac in trophoblasts and preeclampsia”

Bezüglich dieser Veröffentlichung wurde mir ebenfalls eine geteilte Erstautorenschaft zusammen mit Dr. Sarah Meister zugeschrieben.

Diese begründete sich in der gemeinsamen Versuchsplanung, sowie dem gemeinsamen Verfassen der ursprünglichen Version des Manuskripts. Zudem führte ich die Experimente sowie deren Auswertung durch. Auch war ich mit der statistischen Analyse und Visualisierung der Ergebnisse anhand von Grafiken und Zellbildern betraut.

Dr. Sarah Meister war neben den bereits genannten gemeinsamen Aufgaben zudem an der Projektplanung und -betreuung beteiligt und war sowohl für die Bereitstellung der Ressourcen als auch die Datenpflege zuständig.

II. Zusammenfassung

Die PE ist eine schwerwiegende Schwangerschaftserkrankung mit hoher maternaler und fetaler Mortalität und Morbidität (9). Auch wenn die Pathophysiologie der PE noch nicht vollends geklärt ist, werden pathologische Prozesse in der Plazenta als ursächlich angenommen. Beteiligt zu sein, scheinen dabei, eine gestörte Trophoblasteninvasion (32), ebenso wie eine fehlerhafte Umwandlung der Spiralarterien (6) und eine kardiovaskuläre Maladaptation der Mutter an den Fetus (43). Auch scheinen entzündliche Prozesse in der Plazenta auf die Pathophysiologie der PE Einfluss zu nehmen (20, 41). Dabei führt beispielsweise ein überaktives Immunsystem zu einer verminderten Immuntoleranz der Mutter gegenüber dem Fetus (29, 42).

Auch wenn symptomatische Therapieansätze – beispielsweise mittels Antihypertensiva – existieren, ist die einzige aktuell bekannte Therapie die Entbindung, die teilweise zu extremer Frühgeburtlichkeit führt (5).

Um eine kurative Therapie finden zu können, muss jedoch zuallererst die Pathophysiologie der PE mit allen beteiligten Prozessen bestmöglich verstanden werden. Um zu diesem Ziel beizutragen, wurden für diese Arbeit verschiedene Einflussfaktoren der PE mittels immunhistochemischer Färbeverfahren und gezielter Zellkulturexperimente identifiziert.

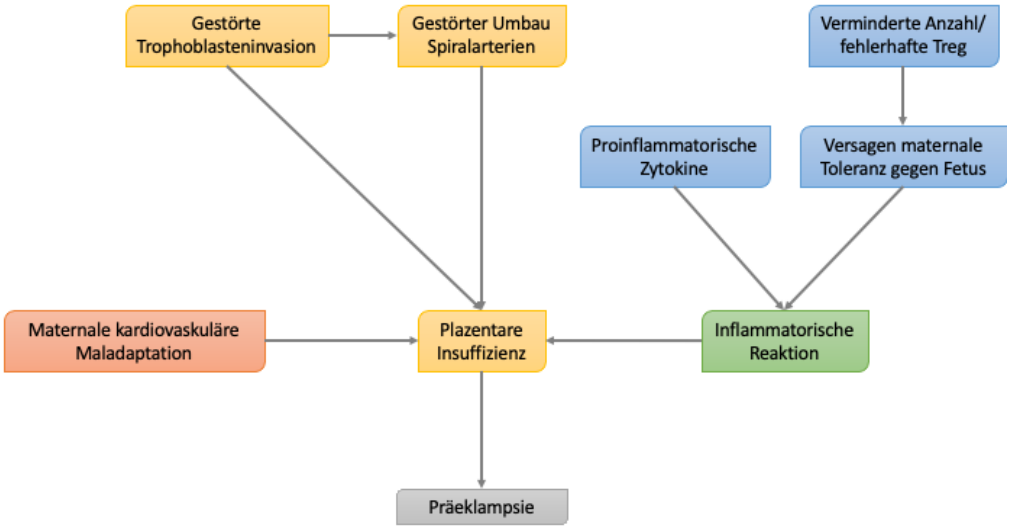

Abbildung 2: Mögliche pathophysiologische Ursachen für die Entwicklung einer Präeklampsie

Es gibt einige Hinweise auf eine epigenetische Beteiligung an der Präeklampsie. So zeigt sich beispielsweise ein dauerhaft gesteigertes Krankheitsrisiko – für Mütter und Kinder, die an einer PE erkrankt waren – für verschiedenste Erkrankungen mit bekannten epigenetische Einflüssen (34, 52). Zudem finden sich Hinweise in der Literatur, beispielsweise eine herabregulierte Genexpression in PE-Plazenten durch

Histonmodifikationen (40, 48). Ähnliche Ergebnisse konnten wir für die von uns untersuchten transkriptionsfördernden Histonmodifikationen H3K4me^3 und H3K9ac nachweisen, die sich in PE-Plazenten vermindert zeigten (1).

Um die Histonmodifikationen H3K4me^3 und H3K9ac bezüglich möglicher Therapieansätze besser beurteilen zu können, wurden weiterführende Experimente zur Beeinflussbarkeit der Histonmodifikationen in Zellkulturexperimenten mit Trophoblasten-Modell-Zelllinien durchgeführt. Ausgewählt wurden für dieses Experiment zwei Proteine, die bekanntermaßen in der PE vermindert sind: PPARγ und Gal-2. Für beide Proteine fanden sich sowohl in der Literatur als auch durch positive Korrelationen der immunhistochemischen Färbeergebnisse Hinweise auf einen Zusammenhang mit den Histonmodifikationen H3K4me^3 und H3K9ac. Dabei führte die Zugabe eines PPARγ-Agonisten zu einer verminderten Expression von PPARγ und den Histonmodifikationen, während der PPARγ-Antagonist die Expression beider erhöhte. Im Fall von Gal-2 konnte eine konzentrationsabhängige Erhöhung der Histonmodifikationen H3K4me^3 und H3K9ac nachgewiesen werden. Zudem konnten wir zeigen, dass die Zugabe von Gal-2 zu einer erhöhten Synzytialisierung der Trophoblasten-Modell-Zellen führte (2, 4). Die Modulation von PPARγ und Gal-2 scheinen somit die Mechanismen der Plazentation zu beeinflussen.

Neben der gestörten Plazentation stellt die fehlgeleitete Immunregulation einen der großen Pfeiler der Pathophysiologie der PE dar (29, 41). Treg – die einen relevanten Einfluss auf den Erhalt des semi-allogenen Fetus haben – sind bekanntermaßen in der PE vermindert, jedoch ist die Ursache für diese Verminderung bisher unerforscht (13, 106, 110, 111). Unser Ziel war es daher, die Verbindung der Treg mit CCL22, das bekanntermaßen Treg rekrutiert (121, 123), in der PE zu untersuchen. Darauf aufbauend sollte in einem weiteren Schritt eine mögliche Ursache der verminderten Anzahl der Treg geklärt werden.

Obwohl es uns möglich war, eine verminderte Anzahl an Treg in PE-Plazenten im Vergleich zu Kontrollplazenten nachzuweisen, fanden wir interessanterweise eine erhöhte Expression von CCL22 in PE-Plazenten. Nichtsdestotrotz fand sich eine positive Korrelation zwischen der Anzahl an Treg und der Expression von CCL22, weshalb wir in unserer Arbeitshypothese nicht von einer fehlerhaften Rekrutierung der Treg, sondern von einer vermehrten Apoptose der Treg ausgingen, welche wir anhand einer TUNEL-Färbung nachweisen konnten. Um diese Erkenntnis therapeutisch nutzbar zu machen beschlossen wir in einem Zellkulturexperiment mit frisch isolierten Treg Möglichkeiten zu erforschen, um Apoptose der Treg zu verhindern.

Aufgrund ihrer immunmodulierenden Wirkung entschlossen wir uns, die Proteine der Galektin-Familie näher zu betrachten und wählten schließlich Gal-2 für unser Experiment aus. Zum einen, da es ein bekanntermaßen in der PE erniedrigtes Protein ist, zum anderen da die Auswertung der immunhistochemischen Färbungen eine positive Korrelation zwischen der Expression von Gal-2 und der Anzahl an Treg lieferte. Die protektive Wirkung von Gal-2 auf die Treg konnte nach FasL-mediierter Apoptose durch den Nachweis einer verringerten Konzentration aktiver Caspase-3 bei Zugabe von Gal-2 bewiesen werden. (3)

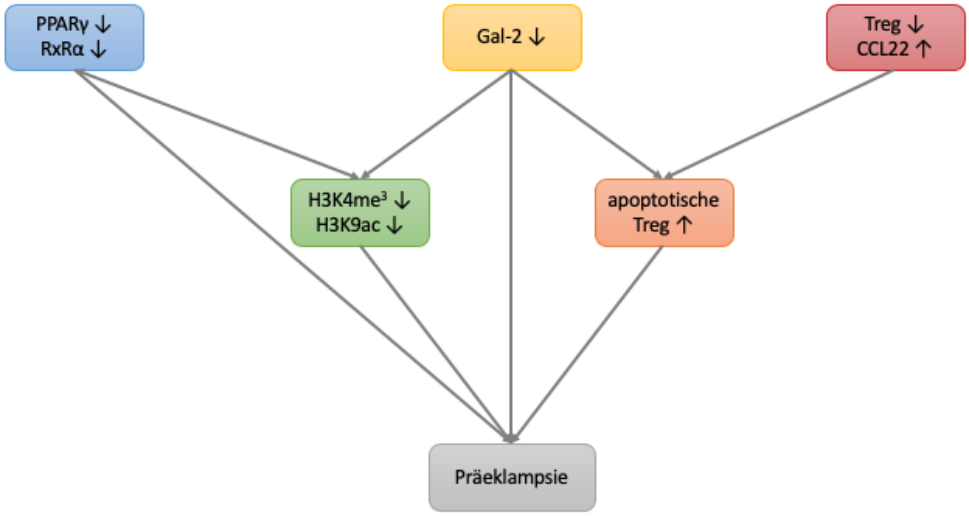

Abbildung 3: Zusammenfassende Darstellung der untersuchten Proteine und Zellen und deren Zusammenhänge in der Präeklampsie.

Zusammenfassend lassen sich die folgenden Schlussfolgerungen ziehen: Die Histonmodifikationen H3K4me^3 und H3K9ac sind in PE-Plazenten vermindert und verdeutlichen somit die Relevanz epigenetischer Veränderungen in der Präeklampsie (1). Zudem konnten wir zeigen, dass PPARγ und Gal-2 einen Einfluss auf die Histonmodifikation in Trophoblasten zeigt (2, 4). Des Weiteren postulieren wir, dass die Stimulation der Synzytialisierung durch Gal-2, in Zusammenhang mit H3K4me^3 beziehungsweise H3K9ac stehen könnte (4). Darüber, ob die Histonmodifikationen tatsächlich an der Pathogenese der PE beteiligt sind oder nur aus anderen Faktoren resultieren, können wir jedoch keine finale Aussage treffen.

Treg scheinen während der PE vermehrt Apoptose zu durchlaufen. Dies – in Zusammenhang mit der erhöhten Expression von CCL22 in PE-Plazenten – könnte auf eine positive Rückkopplungsschleife hinweisen, die ein interessantes zukünftiges Forschungsthema darstellen könnte. Unsere Forschung zeigt, dass die Apoptose von Treg durch Gal-2 verhindert werden kann und somit Gal-2 einen potenziellen therapeutischen Angriffspunkt darstellen könnte (3).

Um aus der Zusammenschau unserer Ergebnisse tatsächlich ursachenspezifische therapeutische Ansätze entwickeln zu können, ist zwar weitere Forschung nötig, jedoch scheint insbesondere Gal-2 ein vielversprechendes Protein darzustellen. Weitere Forschung könnte sich dabei beispielsweise zunächst mit der Applikation von Gal-2 in in-vitro Modellen der Präeklampsie oder im Tierversuch sowie der Möglichkeit Gal-2 zu induzieren, beschäftigen.

III. Abstract (Englisch)

PE is a severe pregnancy disorder with high maternal and fetal mortality and morbidity (9). Although the pathophysiology of PE is not completely understood, pathological processes in the placenta are considered to be causative. Impaired trophoblast invasion seems to be involved (32), as well as defective remodeling of the spiral arteries (6) and maternal cardiovascular maladaptation (43). Furthermore, inflammatory processes in the placenta seem to be involved in the pathophysiology of PE (20, 41). For example, an overactive immune system leads to decreased immune tolerance of the mother to the fetus (29, 42). Although there are symptomatic therapeutic approaches - for example, by using anti-hypertension drugs - the only known treatment is the delivery, which, however, sometimes leads to extreme prematurity (5). To be able to find a curative therapy, first of all the pathophysiology of PE with all involved processes must be understood better. To contribute to this goal, several factors influencing PE were identified in this work using immunohistochemical staining techniques and selected cell culture experiments.

Evidence for epigenetic involvement in PE exists already. For example, there is a permanently increased risk for mothers and children who suffered from PE for several diseases with known epigenetic influences (34, 52). In addition, the literature indicates a down-regulated gene expression in PE placentas by histone modifications (40, 48). We demonstrated similar results for the transcription-promoting histone modifications H3K4me^3 and H3K9ac, which we investigated and which were found to be decreased in preeclampsia placentas (1). To better evaluate the histone modifications H3K4me^3 and H3K9ac with respect to potential therapeutic approaches, further experiments on the influenceability of histone modifications were performed in cell culture experiments with trophoblast model cell lines. Two proteins known to be decreased in preeclampsia were selected for this experiment: PPARγ and Gal-2. For both proteins, evidence for a relationship with histone modifications H3K4me^3 and H3K9ac was found both in the literature and by positive correlations of immunohistochemical staining results. In this regard, the addition of a PPARγ agonist resulted in decreased expression of PPARγ and the histone modifications, whereas the PPARγ antagonist increased the expression of both (2). In the case of Gal-2, a concentration-dependent increase in the histone modifications H3K4me3 and H3K9ac was demonstrated. In addition, we demonstrated that the addition of Gal-2 resulted in increased syncytialization of trophoblast like cells (4). Thus, modulation of PPARγ and Gal-2 appear to influence the mechanisms of placentation.

In addition to impaired placentation, aberrant immune regulation represents one of the major contributors to the pathophysiology of preeclampsia (29, 41). Treg – which have a relevant impact on the maintenance of the semi-allogenic fetus – are known to be decreased in PE (13, 106, 110, 111), but the cause of this decrease has remained unexplored. Therefore, our aim was to investigate the association of Treg with CCL22 – which is known to recruit Treg (121, 123) – in PE and, based on the result, to attempt to determine the cause of the decrease in Treg.

Although we were able to detect a decreased number of Treg in PE placentas compared with control placentas, interestingly, we found an increased expression of CCL22 in PE placentas. Nevertheless, we found a positive correlation between the number of Treg and the expression of CCL22. Therefore, our working hypothesis was not based on a defective recruitment of Treg, but on an increased apoptosis of Treg, detected by TUNEL staining. In order to use this finding for therapeutic purposes, we decided to explore possibilities to prevent Treg apoptosis in a cell culture experiment with freshly isolated Treg. Because of its effect on immune cells, we decided to analyse the galectin family more in detail and finally chose Gal-2 for our experiment, since it is known to be decreased in PE and it provided an indication of correlations with the number of Treg in the evaluation of the immunohistochemical staining. The protective effect of Gal-2 on Treg was demonstrated after FasL-mediated apoptosis by demonstrating a decreased concentration of active caspase-3 upon addition of Gal-2. (3)

In summary, the following can be stated: The histone modifications H3K4me^3 and H3K9ac are decreased in PE placentas, indicating the relevance of epigenetic changes in PE. In addition, we demonstrated that PPARγ and Gal-2 show an influence on histone modification in trophoblasts. In addition, we postulate that the stimulation of syncytialization by Gal-2, could be mediated by H3K4me^3 and H3K9ac. However, we are not able to make a final statement on whether the histone modifications are actually involved in the pathogenesis of preeclampsia or result from other factors. Treg appear to undergo increased apoptosis during PE. This, together with the increased expression of CCL22 in PE placentas, might indicate a positive feedback loop that could be an interesting future research topic. Our research demonstrated that apoptosis of Treg can be prevented by Gal-2 and thus Gal-2 could be a potential therapeutic target. (1-4)

Although further research is needed to develop cause-specific therapeutic approaches from the overall picture of our results, Gal-2 in particular seems to be a

promising protein. Further research could, for instance, initially address the administration of Galectin-2 in in vitro models of PE or in animal experiments. Further the possibility to induce Gal-2 in trophoblasts would be an interesting starting point.

IV. Paper I

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Epigenetic modification via H3K4me3 and H3K9ac in human placenta is reduced in preeclampsia

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ABSTRACT

Background: Alterations of DNA accessibility and chromatin structure are associated with diseases. We aimed to investigate epigenetic modifications in preeclampsia (PE), a pregnancy-associated hypertonic disease. Specifically, we addressed histone modification proteins H3K9ac (acetylated lysine 9 of the histone H3) and H3K4me3 (trimethylated lysine 4 of the histone H3) in PE.
Methods: We analyzed expression of histone proteins H3K4me3 and H3K9ac in 32 PE and 32 control placentas by immunohistochemistry. Further, we carried out confirmatory western blot analysis of respective proteins in 6 representative placentas. We then applied regression models with additional adjustment for potential confounders.
Results: Expression of H3K4me3 and H3K9ac is reduced in PE placentas as demonstrated by immunohistochemical stainings and western blot. There are no differences between female and male fetuses in the presence of these histone modifications. H3K4me3 positively correlated with maternal age (r =0.444, p =0.034).
Conclusion: Expression of the placental histone proteins H3K4me3 and H3K9ac is reduced in PE, and independent of fetal gender. Our study underlines the involvement of epigenetic changes in the placenta of women suffering from PE.

1. Introduction

Preeclampsia (PE) is a severe pregnancy complication, affecting 2–5 % of all pregnancies (Apicella et al., 2019). Belonging to the complex of hypertensive disorders, PE is related to a high morbidity and mortality, causing 70.000 maternal deaths per year (Hypertensive Pregnancy Disorders: Diagnosis and Therapy. Guideline of the German Society of Gynecology and Obstetrics (S2k-Level)). The main diagnostic criteria are a new onset hypertonia (>140/90 mmHg) in combination with proteinuria (>300 mg/24 h) or other organ dysfunction after the 20th week of gestation (Han et al., 2019).

Research over the last years, revealed that immunological factors

Abbreviations: CK7, cytokeratin 7; DAB, chromogenic 3,3'-diaminoenzidine; DAPI, 4',6-Diamino-2-phenylindile; EVT, extravillous trophoblast; H3K4me3, trimethylated lysine 4 of the histone H3; H3K9ac, acetylated lysine 9 of the histone H3; HRP, horseradish peroxidase; IHC, immunohistochemical staining; IRS, Immunoreactive Score of Remmele; IUGR, intrauterine growth retardation; LMU, Ludwig Maximilian University of Munich; PE, preeclampsia (respectively: preeclampsia placentas); PFA, perfluoroalkoxy alkane; PlGF, placental growth factor; PVDF, polyvinylidene difluoride; RIPA, radioimmunoprecipitation assay buffer; RT, room temperature; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor.

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(Staff et al., 2010), a release of inflammatory cytokines (Redman et al., 1999; Benyo et al., 2001) and genetic factors are involved in PE, as well as dysfunctional vascular (Rasmussen et al., 2015) and trophoblastic invasion (Kaufmann et al., 2003). However, the pathogenesis and the molecular mechanisms of PE development are still incompletely understood. Furthermore, there is no cause-specific treatment existing for clinically confirmed PE and merely delivering the baby and thereby the placenta (Irgens et al., 2001) leads to an improvement of the PE following symptoms, morbidity and mortality. Regardless, along with prenatal PE also postnatal PE can occur and affect maternal health considerably leading to severe cardiovascular consequences with only supportive ways of therapy being available (Bellamy et al., 2007; Wikström et al., 2005; Irgens et al., 2001).

Aside from the genetic factors found to be related to PE, epigenetic changes, defined as a variation of gene expression without a change in the DNA sequence (Kamrani et al., 2019a), have been found in the placenta of preeclamptic women. Next to the widely known epigenetic alteration of DNA methylation, histone modifications represent crucial epigenetic changes in PE. As proteins located in the nucleus of eukaryotic cells, histones play an important role in the formation of chromosomes since DNA strands screw around histone proteins and form nucleosomes (Kornberg, 1974). Histones can be modified by acetylation, leading to a more relaxed chromatin configuration and therefore, to an enhanced transcription of genes (Pozharny et al., 2010). Phosphorylation (Patsouras and Vlachoyiannopoulos, 2019) and methylation can induce both, activation and inhibition of gene transcription (Apicella et al., 2019).

In the literature, it is more known about the role of epigenetic modification in the development and differentiation of the placenta compared to other, e.g. immunological mechanisms. A variety of methylated genes are related to PE (Majchrzak-Celinska et al., 2017; Kamrani et al., 2019b), for example, the HSB11B2 gene encoding for 11ß-HSD2 which plays an essential role in hypertension (Friso et al., 2015). Various acetylated lysines of histones, e.g. H3K9, H3K14 and H3K27, have been shown to be reduced by placental ischemia or hypoxia of trophoblast cells (Kamrani et al., 2019a) related to a disturbed angiogenesis and syncytitialization. However, these findings point to a relevance of histone modifications being especially involved in cell differentiation and development during pregnancy and PE development. There are several studies showing an involvement of histone modifications in the regulation of the cellular immune response e.g. in the case of infection (Carson and Kunkel, 2017; Hardbower et al., 2017).

The trimethylated lysine of the histone H3 (H3K4me3) is known to be an activator of the transcription via changing size and hydrophobicity (Lund et al., 2019), next to the important active gene promotor H3K9ac (Karmodiya et al., 2012). Since both histone proteins H3K4me3 and H3K9ac are unknown in PE but have been recently found to be reduced in the placenta of IUGR (intrauterine growth restriction) affected pregnancies (Meister et al., 2020), regarding the several similarities of placental pathology between preeclampsia and IUGR pregnancies, it was of interest to investigate their changes in PE.

2. Methods and material

2.1. Sample – placental tissue

The analyzed tissue was collected at the Department of Obstetrics and Gynecology of the LMU Munich between 2007 and 2019.

The samples consisted of two different collectives with a total number of 32 PE placentas and 32 control placentas. The maternal age at delivery varied between 17 and 44 years (mean =32.20 ±5.704 years). The week of pregnancy varied between 27 and 40 weeks (Supp. Table 1).

Fetal sex is divided into 30 females and 29 males, for 5 placentas the gender declaration was missing. To exclude the week of gestation as a potential confounding factor, a regression analysis was performed (Supp. Figs. 1–4).

After graphical analysis of possible relationships, linear regression was performed for the regression analysis. Since several weeks of pregnancy are not available due to poor documentation and old samples, the result of the regression analysis can only be interpreted to a limited extent. For H3K4me3 neither in the syncytium ($p = 0.219$) nor in the decidua ($p = 0.972$) a significant influence of the weeks of gestation could be shown (Supp. Figs. 1,2). For H3K9ac, no significant effect of the weeks of gestation was shown in the syncytium ($p = 0.083$) either (Supp. Fig. 3). In contrast for H3K9ac week of gestation interfered with the staining results of the decidua ($p = 0.037$), which, can however be only classified as low ($R = 0.276$) (Supp. Fig. 4).

2.2. Immunohistochemical staining (IHC)

Tissue samples were collected immediately after delivery and were fixed in 4% PFA (perfluoroalkoxy alkane). Immunhistochemistry was performed according to a protocol described earlier (Hutter et al., 2015). For the next step the tissue sections were incubated for 16h at 4 °C in a humid chamber in the refrigerator with the primary antibody, anti-H3K4me3-antibody (Abcam, Cambridge, UK), anti-H3K9ac-antibody (Abcam, Cambridge, UK); followed by the HRP-Polymer (horseradish-peroxidase-Polymer) reagent (ZytoChem Plus HRP Polymer System, mouse/rabbit; Zytomed Systems, Berlin, Germany) 30 min at room temperature (RT) (Table 1).

Visualization by the chromogenic 3,3'-diaminoenzidine(DAB; Dako, Glostrup, Denmark) followed, the staining reaction was stopped after a specific period for each primary antibody (H3K4me3 1 min, H3K9ac 30 s) by adding distilled water. Positive controls for the histone-expression were created by staining sigma- (H3K4me3) and colon-tissues (H3K9ac). As negative control the primary antibody was replaced with the correspondent IgG isotype control serum.

2.3. Evaluation of staining

Immunohistochemical stainings were evaluated using the semi-quantitative Immunoreactive Score of Remmele (IRS). The IRS was calculated by multiplying the staining intensity (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) with the percentage of positively stained cells (0 = no staining, 1 = <10 % of cells, 2 = 11–50 % of cells, 3 = 51–80 % of cells, 4 = >80 % of cells stained). The expression of histone modifications in the placental tissue (syncytiotrophoblast cells and decidua) was analyzed by using the IRS.

2.4. Additional methods

For detailed description of Western blot of frozen placenta tissue and immune fluorescence double staining please see supplemental methods.

2.5. Statistical analysis

For statistical analysis the software SPSS (version 24; IBM company, Chicago, IL) and GraphPad Prism (Version 6.0; GraphPad Software, La Jolla, CA) were used. Due to the not normally distributed variables, non-

Table 1
Antibodies used in the immunohistochemistry (IH), immunofluorescence (IF) and immunocytochemistry (ICH).

Antibody	Species isotype	Manufacturer	Dilution	
H3K9ac	Rabbit IgG monoclonal (Clone: Y28)	Abcam	1:200	IH, IF, ICH
H3K4me3	Rabbit IgG polyclonal	Abcam	1:100	IH, IF, ICH
CK7	Mouse IgG1 monoclonal (Clone: NCL-L-CK7-OVTL)	Novocastra	1:30	IF
Cy2	Goat-Anti-Mouse IgG	Dianova	1:100	IF
Cy3	Goat-Anti-Rabbit IgG	Dianova	1:500	IF

parametric tests were used for statistical analysis. The Mann-Whitney-U-test was used for independent samples and the Wilcoxon-signed-rank-test for paired samples. The results of these tests are given as mean value ± standard deviation. The Spearman-Rho correlation test was used to examine the correlations. The correlation coefficient r indicates the strength of the correlation ($r < 0.3$ weak relation, $r > 0.3$ medium relation, $r > 0.5$ strong relation). For the western blot analysis the unpaired student's T-test was used to examine differences between the two groups. The significance level for all tests was assumed at $p < 0.05$.

3. Results

3.1 Down-regulation of H3K4me3 and H3K9ac in preeclampatic placentas

3.1.1. Western blot of H3K4me3 and H3K9ac in preeclampatic placenta

To find out whether there is a quantitative difference of H3K4me3 and H3K9ac in placental tissue from control patients compared to PE affected mothers, western blot was performed on frozen tissue. A decreased amount of H3K4me3 was found by western blot in PE affected placenta compared to the control (Fig. 1). The ß-actin normalized mean amount of H3K4me3 in PE was reduced (normalized H3K4me3 PE = 0.42 ± 0.32; normalized H3K4me3 control = 1.97 ± 1.95). Same appeared for H3K9ac. There the ß-actin normalized mean amount of protein accounted for 0.44 ±0.31 in PE compared to the control with 1.42 ± 0.82.

3.1.2. Trimethylated histone H3K4me3 is reduced in preeclampsia

The immunohistochemically analyzed expression of H3K4me3 in the syncytiotrophoblast was significantly decreased in PE placentas with an IRS of 5.38 ±1.930 compared to the IRS of 6.72 ±2.691 in control placentas. Correlation analysis was performed to epidemic data of the sample. The expression of methylated histone in the syncytiotrophoblast showed a significant positive correlation to higher maternal age in PE placentas (r =0.444, p = 0.034).

H3K4me3 expression in the decidua was also significantly decreased in PE placentas. Comparing the decidual staining in PE placentas (4.34 ±1.842) and control placentas (5.74 ±2.658) a statistically significant difference of the IRS was detected.

The IRS values of the syncytium (5.38 ±1.930) and decidua (4.34 ±1.842) differed significantly in PE (p =0.041) with a higher expression in the syncytium. Analysis of female and male differences between H3K4me3 staining did not show any significance in decidua and syncytiotrophoblast.

The overall results of the stainings are shown in Fig. 2.

3.1.3. Reduction of acetylated histone H3K9ac in preeclampsia

The IHC analysis of H3K9ac showed similar results to the methylated histone. Histone acetylation in the syncytiotrophoblast was significantly decreased in PE placentas (IRS =3.31 ±1.401) compared to the control placentas (IRS =4.91 ±2.161). The overall results of the staining are shown in the box-plots below(Fig. 3). The expression of H3K9ac in the decidua was significantly lower in PE placentas (IRS =3.31 ± 1.958) compared to controls (IRS =5.19 ±2.101).

In contrast to the methylated histone, no significant difference was found between the mean IRS values of the syncytiotrophoblast and the decidua (neither for the total collective nor within the PE placentas). The values between male and female did not show any significant difference in the staining of H3K9ac neither in the decidua nor in the syncytiotrophoblast.

The analysis of correlations between histone acetylation and maternal epidemic data showed no significant results.

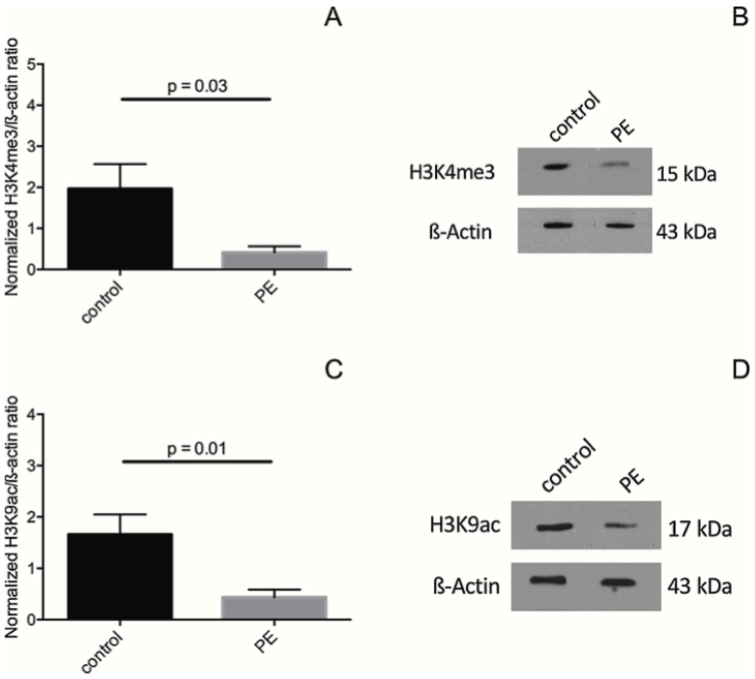

Fig. 1. Western blot of H3K4me3 (A, B) and H3K9ac (C, D) in the placenta tissue. (A) Western blot analysis of H3K4me3 normalized to ß-actin, mean ± SD of control and PE placentas, p values were calculated with unpaired student's T-test, p = 0.03; (B) western blot protein bands of H3K4me3 and ß-actin in control and PE placentas; (C) Western blot analysis of H3K9ac normalized to ß-actin, mean ± SD of control and PE placentas, p values were calculated with unpaired student's T-test, p = 0.01; (D) western blot protein bands of H3K9ac and ß-actin in control and PE placentas.

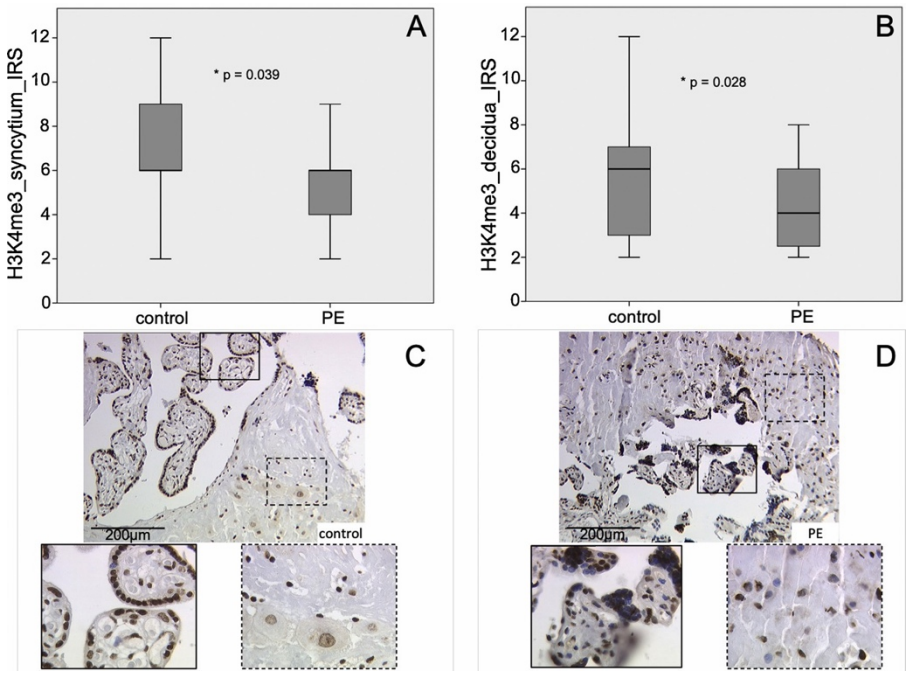

Fig. 2. Staining results of the methylated histone H3K4me3. Boxplots of the IRS in the (A) syncytiotrophoblast and (B) the decidua as mean ± SD; p values were calculated with Mann-Withney-U-Test, $p_{H3K4me3\_syn}$ = 0.039, $p_{H3k4me3\_dec}$ = 0.028; representative immunohistochemical images of H3K4me3 in controls (C) and PE placentas (D) were chosen, continuous line: syncytium, dotted line: decidua. IRS of control placenta: syncytiotrophoblast = 12, decidua = 4; IRS of PE placenta: syncytiotrophoblast = 4, decidua = 6.

3.2. Identification of decidual cells expressing H3K4me3 and H3K9ac

To identify the decidual cell type in which H3K4me3 and H3K9ac are located a double staining with CK7, to stain for extravillous trophoblast (EVT), was performed. Double filter excitation showed a co-expression of H3K4me3 in nuclei of EVT (Fig. 4G, H) in PE placentas, whereas the expression of H3K4me3 in the non-marked decidual tissue was marginal, yet existent. Same was found in the case of H3K9ac (Fig. 4C, D).

4. Discussion

In general, histone modification has been described to be responsible for the regulation of transcription of various proteins (Patsouras and Vlachoyiannopoulos, 2019). Some studies have already pointed to an association of histone modification and preeclampsia. However, intensive research needs to be done in the field of epigenetic changes to contribute to the understanding of the pathophysiology of PE.

In the present study we investigated two histone modifications being known to indicate active regions at gene enhancers and promoters (Wang et al., 2008; Yavartanoo and Choi, 2013). We found a reduction of trimethylated histone H3K4me3 and the acetylated H3K9ac in PE which points to a loss of accessibility of genes to be transcribed.

Histones enable a dense packing of a sizeable amount of DNA very space-saving but simultaneously they leave the N-terminal tails plastic (Iwasaki et al., 2013). The N-terminal tail of the histone proteins can undergo post-translational modification by enzymes, appending chemical modifications that change the structure of the DNA package and allow or prevent gene transcription (Taverna et al., 2007).

A decondensed ("open") configuration allows transcription factors to access to binding sites, whereas a condensed ("closed") configuration blocks transcription binding sites, thereby regulating gene transcription (Yu et al., 2010; Jaenisch and Bird, 2003).

Different histone modifications have been shown to be involved in mechanisms resembling processes happening in PE. For example, an angiotensin II generating enzyme was detected to be increased in PE, regulated via histone modification, whereas an inhibition of histone deacetylase led to an increased angiotensin II production (Gu et al., 2015). Furthermore, an influence of histone modification was found on specific growth factors, which are reduced in PE. Vascular endothelial growth factor (VEGF) shows abnormal trimethylation of H3K9 in the promoter region (Rahat et al., 2017), while changes in histone acetylation of H3 and H4 in the promoter region caused by hypoxia affect placental growth factor (PlGF) (Tudisco et al., 2014).

More importantly, histone modification has been shown to regulate factors which are important for trophoblast invasion and migration which is defective in PE (Apicella et al., 2019; Kamrani et al., 2019a; Staff et al., 2010).

Our data show that the trimethylated histone H3K4me3 and the acetylated H3K9ac are reduced in PE placentas compared to control placentas. The immunohistochemical staining of H3K4me3 and H3K9ac was reduced in the syncytium and the decidua. The decidual cells being positive for the respective histone modifications were identified to be EVTs.

Both histone proteins are known to be closely related to syncytialization and, therefore, are expected to be up-regulated or at least constitutively existing after a successful syncytialization process as demonstrated in BeWo cells (Shankar et al., 2015). Since

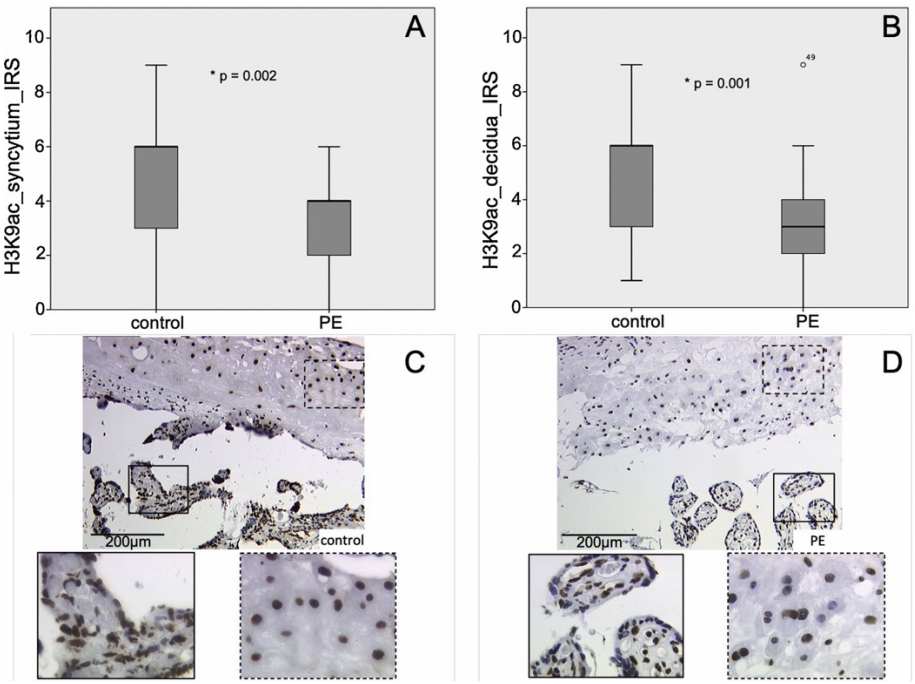

Fig. 3. Staining results of the acetylated histone H3K9ac. Boxplots of the IRS in the (A) syncytiotrophoblast and (B) the decidua as mean ±SD; p values were calculated with Mann-Withney-U-Test, p_{H3K9ac_syn} = 0.002, p_{H3K9ac_dec} = 0.001; representative immunohistochemical images of H3K9ac in controls (C) and PE placentas (D) were chosen, continuous line: syncytium, dotted line: decidua. IRS of control placenta: syncytiotrophoblast =6, decidua =6; IRS of PE placenta: syncytiotrophoblast =4, decidua =4. The circle in B symbolizes an outlier value.

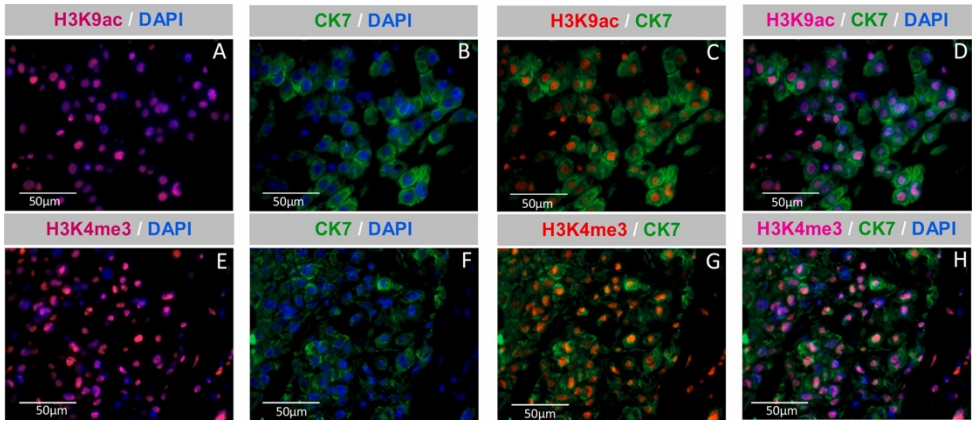

Fig. 4. Immunofluorescence staining of CK7 with H3K9ac (A-D) and H3K4me3 (E-H) in the decidua of PE placentas. Single immunofluorescence of H3K9ac with DAPI in PE placenta (A), single immunofluorescence of CK7 with DAPI in PE placenta (B and F); double immunofluorescence of H3K9ac and CK7 in PE placenta (C), merge of H3K9ac and CK7 in PE placenta with DAPI as nucleus staining (D); single immunofluorescence of H3K4me3 with DAPI in PE placenta (E), double immunofluorescence of H3K4me3 and CK7 in PE placenta (G), merge of H3K4me3 and CK7 in PE placenta with DAPI as nucleus staining (H).

syncytialization is reported as a precondition of crucial importance for sufficient placentation (Knabl et al., 2015), these findings are coherent with processes involved in the pathophysiology of PE.

Although there are several sex-specific differences reported in PE, e. g. higher levels of pro-angiogenic markers in male foetuses (Muralimanoharan et al., 2013) and higher incidence of pregnancy-induced hypertension in female foetuses (Shiozaki et al., 2011), interestingly, the present study could not show a sex-specific variation of the

investigated histone modifications in PE, which stands in contrast to our previous findings in IUGR placentas (Meister et al., 2020).

In general, H3K9ac represents a well-known histone modification in terms of rapid gene activation. Together with other regulatory functions H3K9ac plays a role in the expression of vascular endothelial cadherin, which has been shown in human endothelial cells to be responsible for sufficient angiogenesis (Shu et al., 2015). A defective or inadequate vascular dilation and remodeling of spiral arteries displays a major role in the pathological mechanisms being involved in PE, conducting to a dysfunctional uteroplacental perfusion and the release of factors which lead to a systemic inflammation (Staff et al., 2010). Additionally, to the findings about vascular remodeling and syncytitialization related to the investigated histone modifications, methylation of H3K4 and acetylation of H3K9 were found to be associated with an activation and polarization of macrophages in the case of mucosal inflammation (Hardbower et al., 2017). Histone deacetylase inhibitors have also been shown to improve immune suppressive function of regulatory T cells in bowel disease of mice (Tao et al., 2007). Therefore, dysregulation of the immune response, widely known as an important component of the pathophysiology of PE, potentially already in a very early phase of pregnancy, might be associated to histone modification. Yet, further investigations are needed to address this question in more detail.

In summary, a decrease of the histone proteins H3K4me3 and H3K9ac could be identified in EVT of PE affected placentas. These findings point towards an epigenetic change, which might be responsible for a reduced gene activation in PE. Since there are multiple associations between the considered histone modifications and parts of the pathophysiologic mechanisms of PE, it would be interesting to find out more about the connection between H3K4me3/H3K9ac and pathophysiologic processes in PE.

Furthermore, limitations of this study are left to be discussed: the lower gestational age in the group of PE which, however, was mostly found not to be statistically associated, needs to be considered when interpreting the results, as well as the missing information about the week of gestation in 5 cases of PE placentas. As this study only describes the reduction of H3K4me3 and H3K9ac in PE, but could not prove a reduced gene activation or a clear connection to pathophysiologic mechanisms, there needs to be done further investigations to clarify these questions. The finding of a significant correlation of methylated histone H3K4me3 with maternal age in PE could point out an association of H3K4me3 to the maternal age in PE.

In summary, fetal sex-independent decrease of the histone proteins H3K4me3 and H3K9ac could be identified. Whether histone modification contributes to the pathogenesis of preeclampsia or factors resulting from preeclampsia lead to the epigenetic changes remains to be elucidated in further explanatory approaches. Potential associations with an altered immune response are possible explanations and will be topic of future investigations.

Our present study underlines the involvement of epigenetic changes in the placenta of women suffering from PE. Further research on this topic also in regard to long-term sequelae of newborns and the respective mothers suffering from preeclampsia are required.

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Declaration of Competing Interest

Sv.M: Research support, advisory board, honoraria and travel expenses from AbbVie, AstraZeneca, Clovis, Eisai, GlaxoSmithKline, Medac, MSD, Novartis, Olympus, PharmaMar, Roche, Sensor Kinesis, Teva, Tesaro; TK: holds stock of Roche, relative employed at Roche; TMK: holds stock of Roche, employed at Roche

This study is part of the doctoral thesis of Laura Hahn.

CRediT authorship contribution statement

Sarah Meister: Conceptualization, Methodology, Writing - original draft. **Laura Hahn**: Investigation. **Susanne Beyer**: Software. **Christina Kuhn**: Validation, Visualization. **Magdalena Jegen**: Formal analysis. **Viktoria von Schönfeldt**: Validation. **Stefanie Corradini**: Validation. **Christian Schulz**: Data curation. **Theresa Maria Kolben**: Formal analysis. **Anna Hester**: Validation. **Tamara Appelt**: Formal analysis. **Sven Mahner**: Writing - review & editing. **Udo Jeschke**: Conceptualization, Supervision. **Thomas Kolben**: Conceptualization, Project administration.

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Appendix A. Supplementary data

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V. Paper II

International Journal of Molecular Sciences
Article

Regulation of Epigenetic Modifications in the Placenta During Preeclampsia: PPARγ Influences H3K4me3 and H3K9ac in Extravillous Trophoblast Cells

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Abstract: The aim of this study was to analyze the expression of peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor α (RxRα), a binding heterodimer playing a pivotal role in the successful trophoblast invasion, in the placental tissue of preeclamptic patients. Furthermore, we aimed to characterize a possible interaction between PPARγ and H3K4me3 (trimethylated lysine 4 of the histone H3), respectively H3K9ac (acetylated lysine 9 of the histone H3), to illuminate the role of histone modifications in a defective trophoblast invasion in preeclampsia (PE). Therefore, the expression of PPARγ and RxRα was analyzed in 26 PE and 25 control placentas by immunohistochemical peroxidase staining, as well as the co-expression with H3K4me3 and H3K9ac by double immunofluorescence staining. Further, the effect of a specific PPARγ-agonist (Ciglitazone) and PPARγ-antagonist (T0070907) on the histone modifications H3K9ac and H3K4me3 was analyzed in vitro. In PE placentas, we found a reduced expression of PPARγ and RxRα and a reduced co-expression with H3K4me3 and H3K9ac in the extravillous trophoblast (EVT). Furthermore, with the PPARγ-antagonist treated human villous trophoblast (HVT) cells and primary isolated EVT cells showed higher levels of the histone modification proteins whereas treatment with the PPARγ-agonist reduced respective histone modifications. Our results show that the stimulation of PPARγ-activity leads to a reduction of H3K4me3 and H3K9ac in trophoblast cells, but paradoxically decreases the nuclear PPARγ expression. As the importance of PPARγ, being involved in a successful trophoblast invasion has already been investigated, our results reveal a pathophysiologic connection between PPARγ and the epigenetic modulation via H3K4me3 and H3K9ac in PE.

Keywords: preeclampsia; histone modification; H3K4me3; H3K9ac; PPARγ; RxRα; placenta

1. Introduction

Affecting around five percent of all pregnancies, preeclampsia (PE) represents one of the most frequent gestational diseases [1] and a relevant cause for maternal deaths [2–4]. PE is defined as new-onset hypertension in pregnancy (>140/90 mm Hg) combined with

organ dysfunction after the 20th week of gestation, most commonly in the sense of pathologic proteinuria (>300 mg/L) [5].

The pathophysiology of PE is currently not completely understood. Additionally, inflammatory processes [1,6–8] and loss of maternal tolerance to the fetus [9–11], maternal cardiovascular maladaptation [12,13] and placental insufficiency [14] appear to be involved in the development of PE. Impaired trophoblast invasion [15], impaired remodeling of spiral arteries [5,16], and defective placentation [13,17–19] contribute to the aforementioned placental insufficiency.

The process of healthy placentation is complex and contains not only vascular remodeling but a complicated process of cell differentiation and cell growth. Considering placental maturing, it is necessary to distinguish between the development of the placental villi (cytotrophoblast proliferation, syncytial fusion) [20] and the trophoblast invasion happening simultaneously to the vascular remodeling [21,22]. The trophoblast invasion begins before the 6th week of pregnancy and happens at the basal plate and the placental bed. All trophoblasts, which reside outside the placental villi are summarized under the term EVT [21]. These trophoblasts migrate together with uteroplacental arteries into the decidua, and it is already known that several instances, such as trophoblast apoptosis, lead to a defective trophoblast invasion in PE [23–25].

Peroxisome proliferator-activated receptors (PPARs), are transcription factors and members of the ligand-activated nuclear hormone receptor superfamily, being involved in energy metabolism, inflammation, and cell development [26–29]. After ligand binding, PPARs form heterodimers with retinoid X receptors (RXRs) and bind to PPAR-response elements (PPREs) of their target genes to activate transcription [26].

PPARγ and RxRα are predominantly known for their important role in adipogenesis and metabolism [27,28]. They are furthermore involved in trophoblast differentiation [30], placentation [31], and differentiation of the syncytium [32]. It has been shown that PPARγ stimulates trophoblast proliferation [33] and plays an important role in trophoblast invasion [34,35]. A lack of PPARγ leads to placental defects [36,37], an increase of proinflammatory cytokines [38] is associated with hypertension [36,39]. Placental PPARγ is produced by trophoblasts and endometrial stromal cells [30,40,41] and is known to be reduced in preeclampsia [32,42].

Even though PPARs are nuclear receptors, there is some evidence for an additional cytoplasmic expression [42–45]. The role of this cytoplasmic expression has not yet been definitively determined, which is why we focused on nuclear PPARγ expression.

Various epigenetic changes have been detected in PE-affected pregnancies such as DNA methylation, non-coding RNAs, and genomic imprinting [46]. Histone modification is a further epigenetic alteration. Histones can be modified by acetylation, methylation, or phosphorylation [47] adjusting the accessibility of the DNA which is wrapped around the histones [14].

Histone modification has been shown to regulate factors that are important for trophoblast invasion and migration which is defective in PE [1,46,48]. Recent data of our group showed a decrease of trimethylated lysine 4 of the histone H3 (H3K4me3) and acetylated lysine 9 of the histone H3 (H3K9ac) in EVT cells of placentas of preeclamptic mothers [49]. As H3K4me3 and H3K9ac are known to affect syncytialisation as a necessary precondition for sufficient placentation [50] there might be involvement of histone modification in trophoblast invasion.

Furthermore, there are associations between PPARγ and histone modifications. In the adipogenesis and late adipocyte differentiation, a positive influence of H3K4me3 and H3K9ac on PPARγ has already been shown [51–53].

Since pathophysiological mechanisms of preeclampsia are not fully elucidated and the disease is hard to investigate, there is no cause-specific therapy except for delivering the baby and the placenta [4,31,54], leading to a higher rate of preterm-births and further morbidities [55]. Several molecular mechanisms still need to be investigated in the pathophysiology of PE, especially controlling gene expression, to hopefully discover a

potential therapeutic target. Therefore, a further investigation of PPARγ and the connection with the histone modifications H3K4me3 and H3K9ac could be illuminating regarding therapy possibilities of PE.

As ineffective trophoblast invasion represents a pivotal element of the pathophysiology of PE, the aim of this study was to analyze the expression of PPARγ and RxRα, especially in the EVT in the placenta of preeclamptic patients, and to characterize a possible association between the histone modifications found to be decreased in the placenta during PE.

2. Results

2.1. Expression of PPARγ and RxRα is Decreased in the Decidua of PE Placentas

PPARγ expression was significantly lower in the decidua of PE placentas 4.56 ± 2.725) compared to controls (7.43 ± 3.727) (p = 0.004) (Figure 1B, C, D). Mean IRS in the syncytiotrophoblast of PE placentas (4.00 ± 2.683) and controls (4.74 ± 3.441) was not significantly different (Figure 1A, C, D).

As PPARγ builds heterodimer complexes with RxRα, the expression of RxRα was analyzed as well. The mean IRS of RxRα in the syncytiotrophoblast and the decidua was significantly diminished in PE placentas (IRS_{syn} = 4.43 ± 2.694; IRS_{dec} = 4.40 ± 2.703) compared to the controls (IRS_{syn} = 6.00 ± 2.322; IRS_{dec} = 7.22 ± 4.011) (p_{syn} = 0.045, p_{dec} = 0.038) (Figure 2A–D).

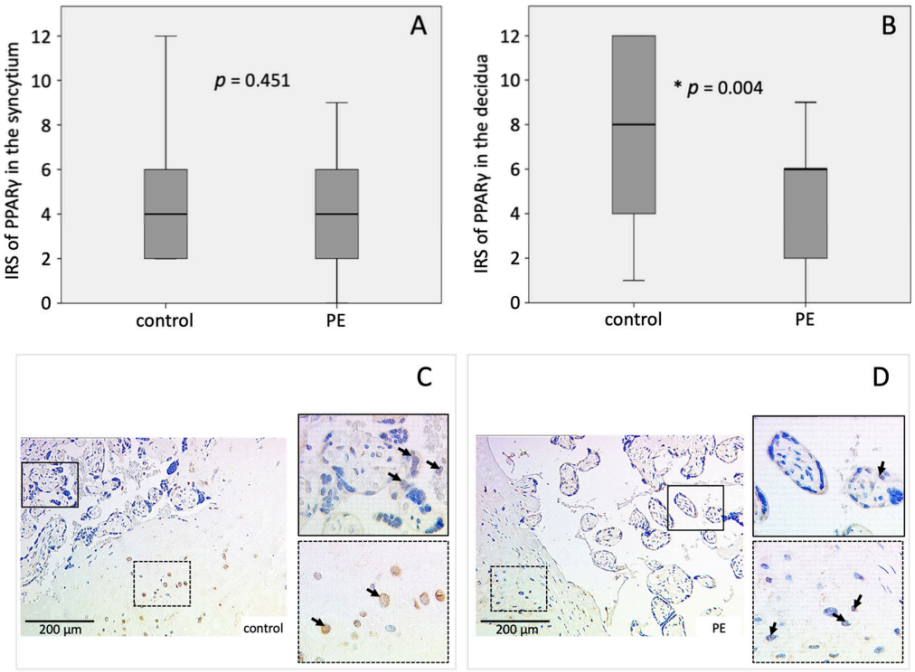

Figure 1. Immunohistochemical staining results of PPARγ. Boxplots of the IRS in the (**A**) syncytiotrophoblast and (**B**) the decidua as mean ± SD; p-values were calculated with Mann–Withney-U-Test; representative immunohistochemical images of PPARγ in controls (**C**) and PE placentas (**D**) were chosen, continuous line: syncytium, dotted line: decidua. IRS of control placenta: syncytiotrophoblast = 4, decidua = 4; IRS of PE placenta: syncytiotrophoblast = 4, decidua = 2. The respective *p*-value indicates, if the IRS of controls and PE differ significantly and was calculated using the Mann-Whitney-U-Test.

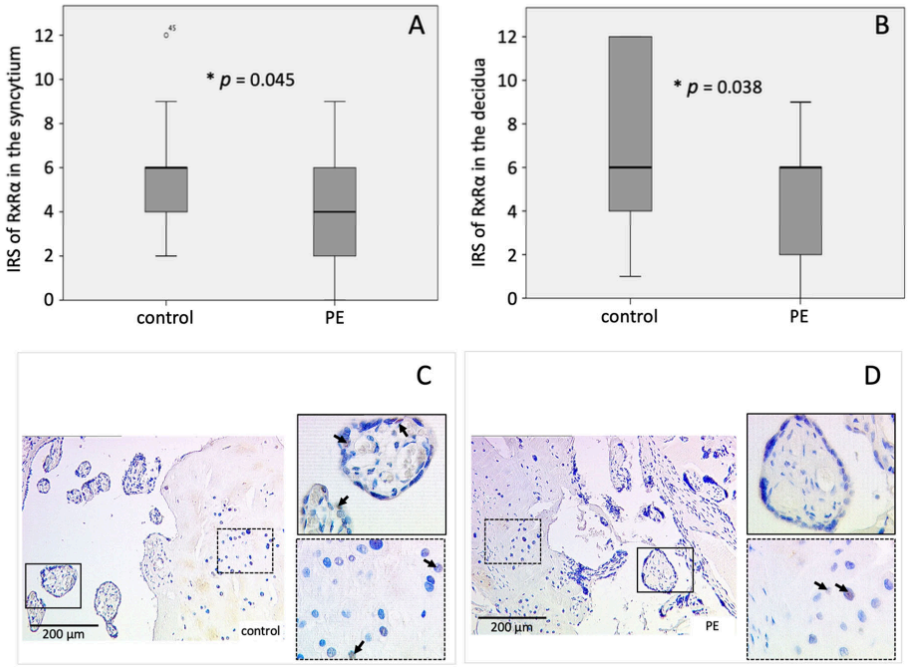

Figure 2. Immunohistochemical staining results of RxRα. Boxplots of the IRS in the (**A**) syncytiotrophoblast and (**B**) the decidua as mean ± SD; p-values were calculated with Mann–Withney-U-Test; representative immunohistochemical images of RxRα in controls (**C**) and PE placentas (**D**) were chosen, continuous line: syncytium, dotted line: decidua. IRS of control placenta: syncytiotrophoblast = 4, decidua = 3; IRS of PE placenta: syncytiotrophoblast = 1 decidua = 2. The respective *p*-value indicates, if the IRS of controls and PE differ significantly and was calculated using the Mann-Whitney-U-Test.

2.2. Correlation of H3K4me3 and H3K9ac with PPARγ and RxRα

A correlation analysis of the IRS of H3K4me3/H3K9ac—published earlier by our group [49] and the expression of PPARγ and RxRα was performed, to outline a possible connection between the transcription modulating PPARγ and RxRα and the histone modifications H3K4me3 and H3K9ac.

Both the acetylated histone H3K9ac as well as the trimethylated histone H3K4me3 correlated significantly positive with RxRα. These significant correlations were found in the syncytium (H3K9ac: r = 0.428, $p = 0.003$; H3K4me3: r = 0.448, $p = 0.002$) as well as in the decidua (H3K9ac: r = 0.464, $p = 0.002$; H3K4me3: r = 0.327, $p = 0.032$).

Furthermore, PPARγ correlated positively with the investigated histone modifications. Significant correlation of H3K9ac and PPARγ in the syncytiotrophoblast (r = 0.284, $p = 0.046$) as well as in the decidua (r = 0.399, $p = 0.004$) were found. Moreover, H3K4me3 in the syncytium correlated significantly positive with PPARγ in the decidua (r = 0.357, $p = 0.012$).

The overall results of the statistical correlation analysis of the histone modifications and PPARγ respectively RxRα are shown in Table 1.

Table 1. Correlations of the histones with other proteins. The upper value is the correlation factor r, the second value is the p-value. * significant ($p < 0.05$), ** highly significant ($p < 0.01$) [56].

	H3K4me3		**H3K9ac**	
	syncytium	decidua	syncytium	decidua
RxRα syncytium	0.448 **	0.513 **	0.428 **	0.577 **
	0.002	0.000	0.003	0.000

RxRα decidua	0.406 **	0.327 *	0.389 **	0.464 **
	0.007	0.032	0.010	0.002
PPARγ syncytium	0.216	0.249	0.284 *	0.379 **
	0.131	0.084	0.046	0.007
PPARγ decidua	0.357 *	0.227	0.347 *	0.399 **
	0.012	0.121	0.014	0.004

2.3. Identification of Decidual Cells Expressing PPARγ/RxRα

To clarify the type of decidual cells expressing PPARγ and RxRα an immunofluorescence double staining with CK 7 was performed. Trophoblasts—in the case of the decidua—EVTs, are expressing CK 7. Therefore, decidual cells stained by CK 7 can be classified as EVTs whereas CK 7 negative tissue cells are considered to be decidual stroma cells.

The immunofluorescence double staining of PPARγ and CK 7 showed a clear double expression in EVTs, in control samples and PE samples (Figure 3A, B).

In a second step, double staining of PPARγ and RxRα was performed. The purpose of this staining was to show the retention of the known heterodimerization of PPARγ and RxRα in preeclamptic placentas (Figure 3C, D).

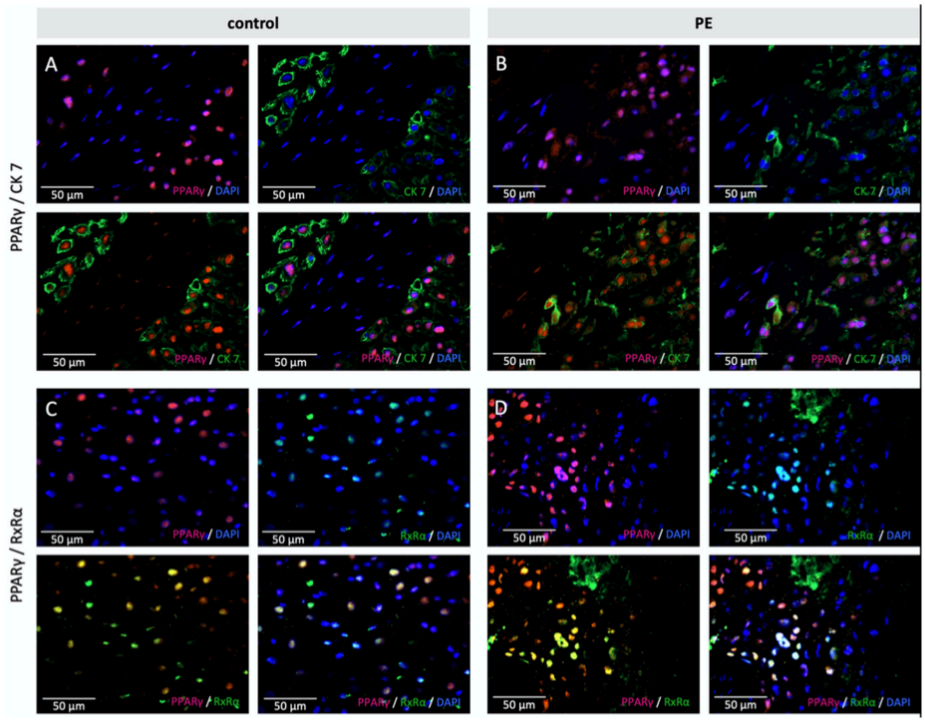

Figure 3. Examples of staining results of the immunofluorescence of PPARγ with CK7 (**A, B**) and RxRα (**C, D**), in control (**A, C**) and PE (**B, D**) placentas. Single immunofluorescence staining of PPARγ (pink). Single immunofluorescence staining CK7 or RxRα (green). Double immunofluorescence staining of PPARγ (**A/B**) and H3K4me3 (**C/D**) (red) and PPARγ (green). DAPI as nucleus staining (blue). Scale bar 100 µm.

2.4. Co-expression of H3K4me3 and H3K9ac with PPARγ and RxRα

Double immunofluorescence staining was performed to verify whether the histone modifications H3K4me3 and H3K9ac are located in the same cell type as PPARγ,

respectively, RxRα. H3K4me3 respectively H3K9ac are presented in red and PPARγ, respectively, RxRα in green. Colocalization is shown as yellow color in the triple filter excitation.

Co-expression appeared in all three analyzed cell types-stromal cells, EVTs and the syncytiotrophoblast, both in control and PE placentas. A distinctly and visibly reduced amount of double-positive cells for H3K9ac respectively H3K4me3 and PPARγ was found in PE (H3K9ac + PPARγ: Control 80–90%, PE 70%, (Figure 4) H3K4me3 + PPARγ: Control 90%, PE 70% (Figure 5)); The difference between control and PE was similar for decidual cells, EVTs, and the syncytium.

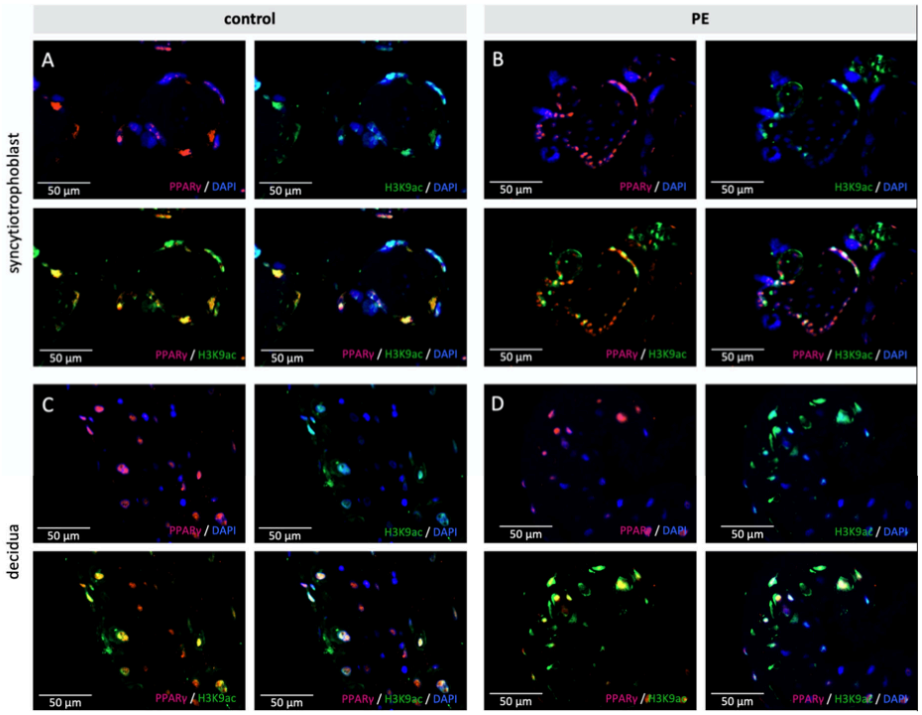

Figure 4. Examples of staining results of the immunofluorescence of PPARγ and H3K9ac (**A, B**)/H3K4me3 (**C, D**), in control and PE placentas. Single immunofluorescence staining of H3K9ac and H3K4me3 (pink). Single immunofluorescence staining of PPARγ (green). Double immunofluorescence staining of H3K9ac (**A/B**) and H3K4me3 (**C/D**) (red) and PPARγ (green). DAPI as nucleus staining (blue). Scale bar 100 µm.

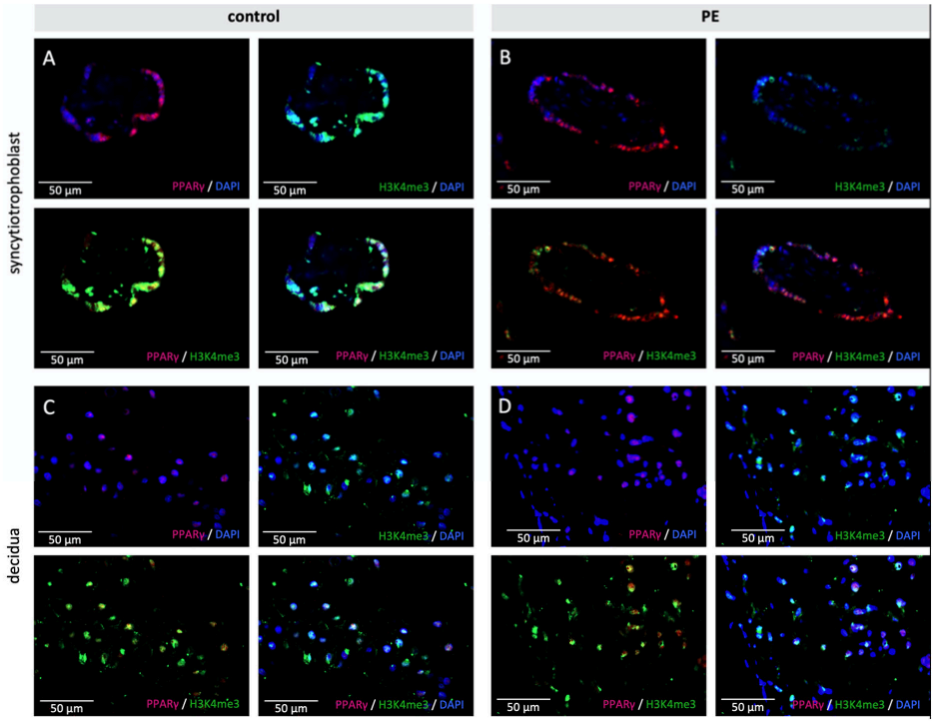

Figure 5. Examples of staining results of the immunofluorescence of PPARγ and H3K4me3 in control (**A, C**) and PE placentas (**B, D**) in the syncytiotrophoblast (**A, B**) and the decidua (**C, D**). Single immunofluorescence staining of PPARγ (pink) and H3K4me3 (green). Double immunofluorescence staining of H3K4me3 and PPARγ (yellow). DAPI as nucleus staining (blue). Scale bar 50 µm.

Further, colocalization of H3K4me3 respectively H3K9ac and RxRα was analyzed. We found a decreased amount of double-stained cells in PE placentas compared to control placentas (Figure S8).

Concerning H3K4me3 (H3K4me + RxRα decidua: Control 40%, PE 10%; H3K4me3 + RxRα syncytiotrophoblast: Control 60%, PE 10%) the effect seemed to be slightly more pronounced in the decidua and syncytiotrophoblast compared to H3K9ac (H3K9ac + RxRα decidua: Control 50%, PE 30%; H3K9ac + RxRα syncytiotrophoblast: Control 50%, PE 30%; H3K9ac + RxRα EVT: Control 80%, PE 60%).

2.5. Specific PPARγ Agonist and Antagonist Regulate H3K4me3 and H3K9ac in Human Trophoblast Cells

To investigate the individual effect of PPARγ on histone modification H3K4me3 and H3K9ac in trophoblasts, the PPARγ agonist Ciglitazone and the PPARγ antagonist T0070907 were used. After 24 h incubation of HVT cells and primary isolated EVT cells with Ciglitazone and T0070907, the expression of histone modifications and PPARγ were evaluated by double immunofluorescence staining. PPARγ expression was reduced in HVT cells after incubation with the PPARγ agonist Ciglitazone and induced by the PPARγ antagonist T0070907 (Figure 5, Figure S9). The mean fluorescence intensity of H3K4me3 was significantly decreased after incubation with Ciglitazone (p = 0.04) and increased after incubation with T0070907 (p = 0.02; Figure 6A, B). The same effect could be detected in the mean fluorescence intensity of H3K9ac ($p_{Ciglitazone}$ = 0.005, $p_{T0070907}$ = 0.004; Figure 6C, D).

For the primary isolated EVT cells, a significant decrease of the mean fluorescence intensity of H3K4me3 and H3K9ac after adding the PPARγ agonist Ciglitazone ($p_{H3K4me3}$ = 0.0143, p_{H3K9ac} = 0.004) could be detected. On the other hand, the PPARγ antagonist T0070907 increased H3K4me3 respectively H3K9ac ($p_{H3K4me3}$ = 0.0065, p_{H3K9ac} = 0.007) in EVT cells(Figure 7A–D).

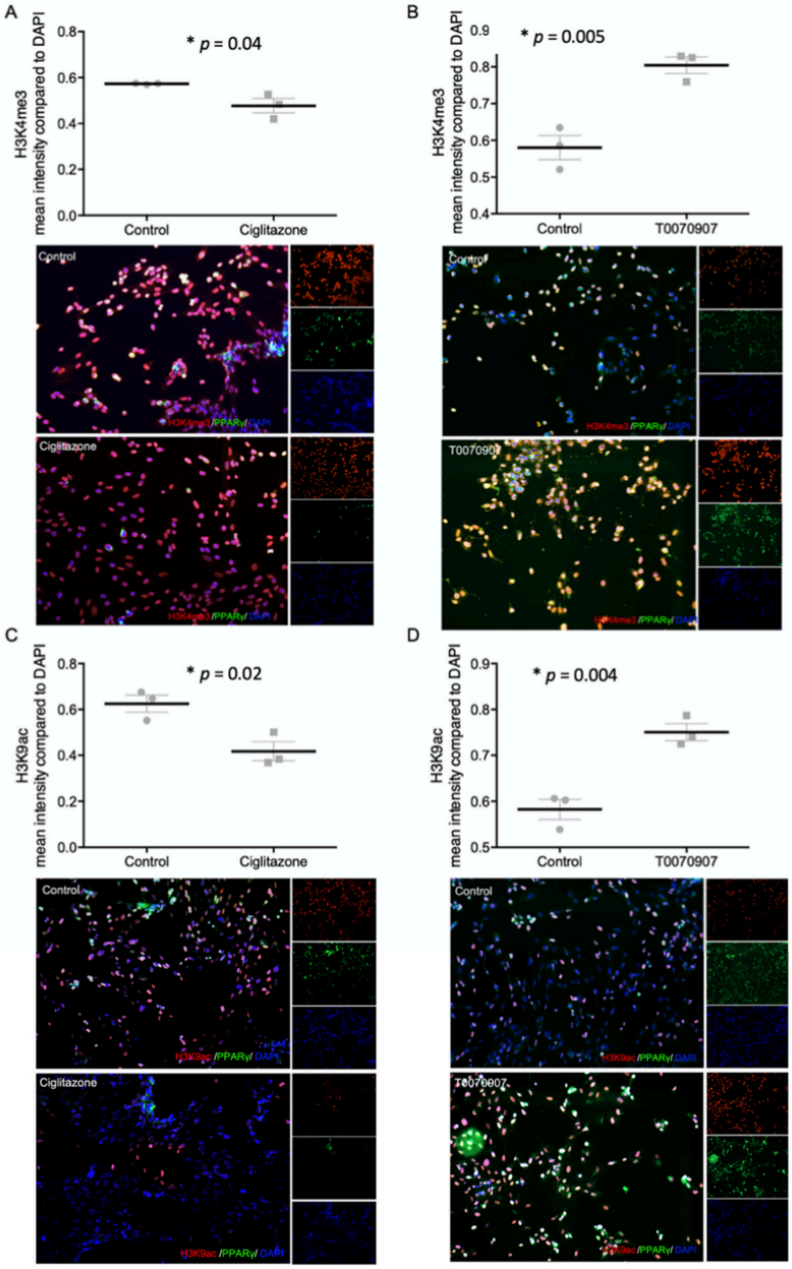

Figure 6. Staining results of histonemodifications H3K4me3 (**A, B**) and H3K9ac (**C, D**) and PPARγ after incubation with Ciglitazone (**A, C**) (20 mM) and T0070907 (**B, D**) (50 mM) mean fluorescence intensity in relation to DAPI, with representative pictures of EVTs, histone modifications shown in red, DAPI as nucleus staining in blue; Dot plots: mean fluorescence intensity ± SEM. The respective p-value indicates, if the intensity of control cells and cells incubated with Ciglitazone or T0070907 differ significantly and was calculated using the Mann-Whitney-U-Test. Images performed with 10× magnification, Scale bar: 50 µm.

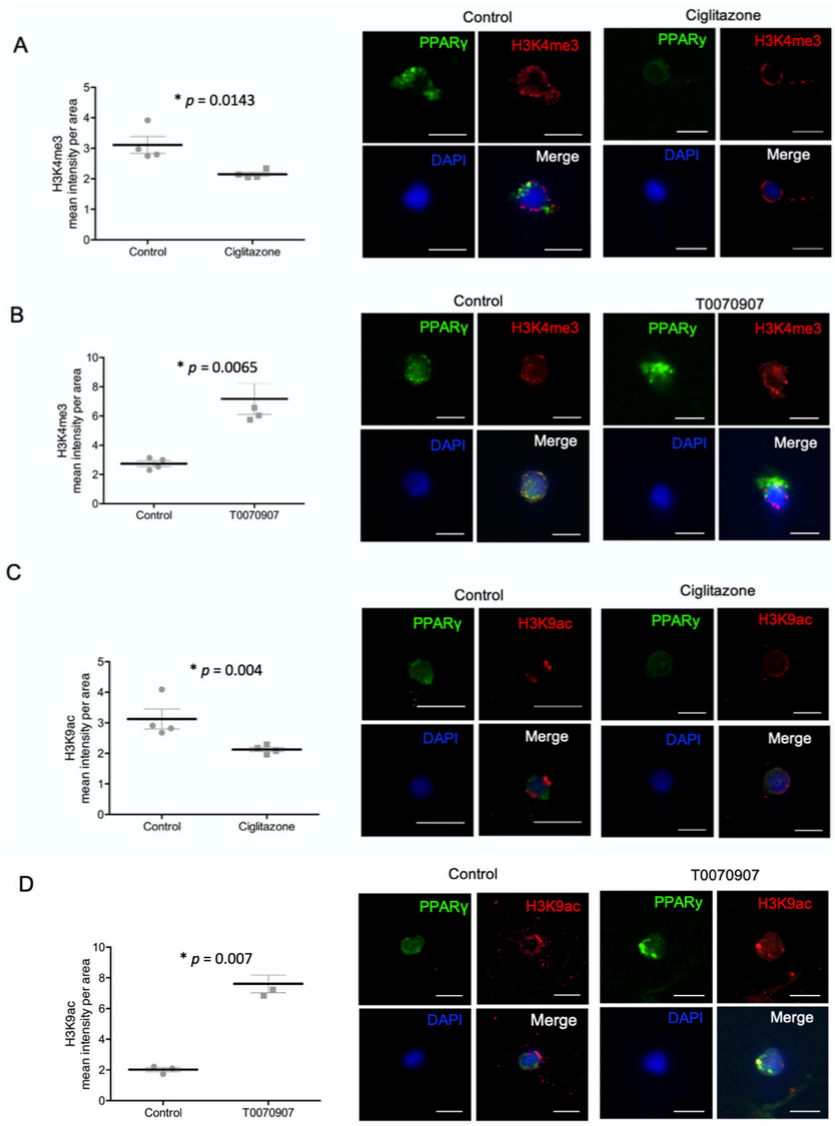

Figure 7. Staining results of histonemodifications H3K4me3 (**A, B**)/ H3K9ac (**C, D**) and PPARγ after incubation with Ciglitazone (**A, C**) (20 mM) and T0070907 (**B, D**) (50 mM) mean fluorescence intensity per area, with representative pictures of isolated EVT cells, histone modifications shown in red, PPARγ in green, DAPI as nucleus staining in blue; Dot plots: mean fluorescence intensity ± SEM. The respective *p*-value indicates, if the intensity of control cells and cells incubated

with Ciglitazone or T0070907 differ significantly and was calculated using the Mann-Whitney-U-Test. Images performed with 10× magnification, Scale bar: 10 μm.

3. Discussion

Several mechanisms are involved in the pathophysiology of PE, such as the release of inflammatory cytokines, genetic predispositions, and immunological imbalance, which complicates the investigation of the underlying basal pathologic processes of this pregnancy disease [48,57]. Especially the dysfunctional remodeling of spiral arteries as well as the defective trophoblast invasion are important factors, that need to be further investigated to elucidate parts of the mechanism [21,48].

Since we have already shown a decrease of the histone modifications H3K4me3 and H3K9ac in PE in a prior publication [49], the aim of this study was to investigate a possible regulation of these histone modifications by proteins that seem to be involved in the pathology of PE. Therefore, we chose PPARγ which is known to play an important role in trophoblast differentiation and invasion [32]. Moreover, a connection of PPARγ with various histone modifications is already known in adipogenesis, and for example, the systemic lupus erythematosus [51,52,58], where a protective effect of PPARγ depending on histone acetylation has been shown [59]. Further, inhibition of histone deacetylase 3 was shown to lead to an increased expression of PPARy [60]. On the other hand, there are findings that point to a possible influence of PPARγ on histone modification, indicated by the experiments of Choi et al. [61] where an inhibitory effect of the histone demethylase KDM4D on adipogenesis could be shown. These effects could be rescued by adding exogenous PPARγ.

One of the most important known roles of PPARγ is the promotion of the transcription by heterodimerization with RxRα [33]. The heterodimer binds to the PPAR-response-element (PPRE) which is composed of two direct-repeat sequences, separated by one or two nucleotides in the promoter region of target genes [62].

In accordance with other publications [32,40], we detected a decreased expression of PPARγ and its binding heterodimer RxRα in the decidual tissue of PE-affected placentas. Additionally, we could detect a regulatory effect of PPARγ on the histone modifications H3K4me3 and H3K9ac. This indicates a pathophysiologic connection between PPARγ and the epigenetic modulation via H3K4me3 and H3K9ac in PE. Further, as PPARγ is implicated as a key regulator of trophoblast differentiation and invasion, which has been concretely shown in mice experiments [35] a decreased expression in the villous trophoblast of PE placentas could indicate defective placentation.

To confirm the association of PPARγ and the histone modifications in preeclampsia, which is known from other diseases, a correlation analysis was performed. The significant positive correlation of PPARγ expression with the histone modifications, as well as the co-expression in EVT, strengthened the idea of a possible regulation of the histone modifications through PPARγ.

To investigate this possible regulation, we performed cell culture experiments with HVT and primary isolated EVT cells. We examined the effect of the PPARγ agonist Ciglitazone and the PPARγ antagonist T0070907 on histone modification by immunocytochemical double immunofluorescence staining of H3K4me3 or H3K9ac and PPARγ.

Interestingly, the agonist Ciglitazone led to a reduced expression of PPARγ and histone modifications, while the antagonist T0070907 led to an increased expression of PPARγ and histone modifications. The interpretation of these results is diverse, but without doubt, a positive association of the expression of PPARγ and histone modifications can be established.

To adequately interpret the results, the in vivo effects of the agonists and antagonists must first be considered.

It has already been shown in animal studies that the inhibition of PPARγ by antagonists, for example, T0070907, leads to reduced fetal growth [36]. In contrast,

activation of PPARy appears to protect against IUGR induced through hypoxia in advanced pregnancy [63]. In addition, as previously shown by Tache et al. [30], treatment with PPARγ antagonists can induce symptoms similar to those in preeclampsia. Moreover, treatment with a PPARγ agonist reduced RUPP-induced hypertension in an animal experiment using the reduced uterine perfusion pressure (RUPP) model [64], which suggests a protective role of PPARγ activity in endothelial cell function. Furthermore, the administration of PPARγ agonist supports villous cytotrophoblast differentiation. On the other hand, blocking PPARγ activation promotes proliferation and prevents differentiation of the villous trophoblast cells [20,65]. According to these findings, a decreased PPARγ-expression in the placental tissue would represent a reduced activation of PPARγ which leads to a defective trophoblast differentiation and invasion. The decreased mRNA level of PPARγ in PE placentas [32], as well as a reduction of circulating PPARγ activation which can be detected weeks to months before diagnosis [40], and our immunohistochemical staining results support this theory.

In contrast to this theory, other findings can be found in the literature, which support a hypothesis deduced from our cell culture results.

Corresponding to our findings, Levtyska et al. [20], who considered the activity of PPARγ in addition to the PPARγ expression, showed that stimulation of PPARγ activity with Rosiglitazone—a selective PPARγ agonist like Ciglitazone—led to a decreased PPARγ expression. Further, inhibition of PPARγ activity with the PPARγ antagonist T0070907 resulted in an increased PPARγ expression.

Barak et al. [66] showed that administration of Rosiglitazone led to changes of placental morphology and reduced the size of the placenta and the spongiotrophoblast layer. Further, EVT cell invasion is inhibited by PPARγ activation and improved by inhibition of PPARγ, shown in different cell culture models [34,67]. In addition, the treatment of mice with Rosiglitazone led to an altered microvasculature in the placenta and to a decreased expression of proangiogenic genes [68].

In summary, there seem to exist different effects of PPARγ stimulation on the different cell types of the placenta and their role during placentation.

PPARγ agonists and antagonists have moreover been studied in other models and biological systems than the placenta. In cancer cell models, inhibitory effects could be demonstrated on cancer cell growth by both, PPARγ agonists and PPARγ antagonists as well as apoptotic effects. [69–71]. These findings show possible rectified effects of PPARγ agonists and antagonists in other cell models.

Our in vivo data underline this hypothesis as we could not find a clear difference of PPARγ-expression in the syncytiotrophoblast. In contrast, in EVT, the PPARγ-expression was decreased. Different studies have already shown an increase of LXRα, the PPARγ-target gene, in the tissue of PE placentas in accordance to an increased PPARγ-activity in PE [72,73]. Therefore, an increased PPARγ-activity might reduce PPARγ expression and vice versa, consistent to our in vitro data.

Prostaglandins which are known to be ligands of PPARγ, being increased in PE [74,75] might have a stimulating effect on the activity of PPARγ in EVTs, but this still needs to be verified in further studies and is merely part of a hypothesis (Figure 8).

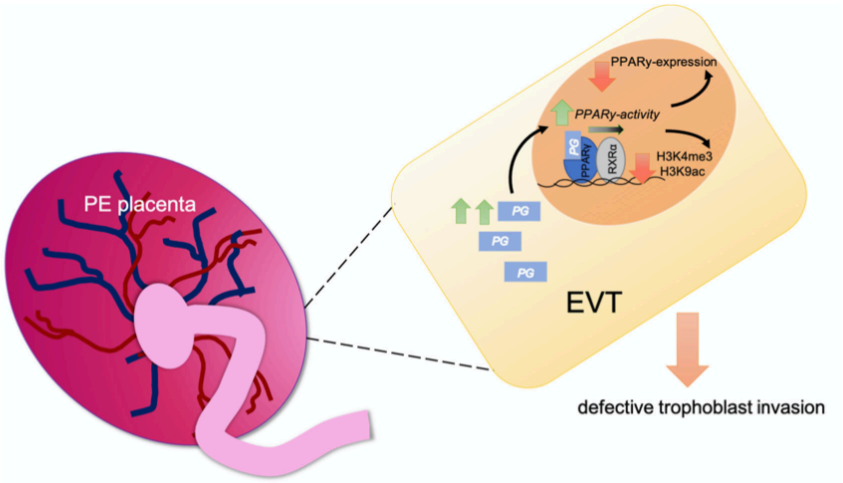

Figure 8. Graphical abstract of the hypothesis of PPARγ regulating H3K4me3 and H3K9ac in EVT (PG (prostaglandin)).

The main limitation of this study is, that PPARγ activity has not been quantified in EVT cells, neither in vivo nor in vitro, and therefore should be part of further examination.

An additional limitation is the lower average week of gestation in the PE group, which needs to be considered in the interpretation of the results, although no statistical association was found.

Furthermore, no clear pathophysiologic statements of the development of PE could be concluded by this study. However, some interesting findings about epigenetic regulation during PE could be contributed.

4. Materials and Methods

4.1. Sample—Placental tissue

A total number of 26 PE placentas and 25 placentas from healthy term pregnancies were analyzed. Mothers at delivery were between 17 and 44 years old (mean = 32.42 ± 5.929 years) and the week of gestation varied between the 25th and 40th (Table S1).

Fetal sex was grouped into 25 females and 20 males, while for five placentas the gender declaration was missing. The equally distributed sex of the newborn was verified. Neither the age of the mother (p = 0.720), nor the sex (p = 0.692), or the weight of the newborn (p = 0.222) differed significantly between the groups of PE and control. Since the week of gestation differed significantly between the two groups (p < 0.001) regression analysis was performed to exclude the week of gestation as a potential confounder' (Figure S1–4).

Placenta tissue was obtained directly after delivery in the Department of Obstetrics and Gynecology, LMU Munich between 2007 and 2019. Classification as preeclampsia was based on the guidelines of the German Society of Gynecology and Obstetrics. The tissue was collected by dissecting a piece of the placenta containing decidua and placental villi. The samples were immediately fixed in 4% neutral buffered formalin for one week, dehydrated, and embedded in paraffin for use in immunohistology and immunofluorescence.

The present study was approved by the local ethics committee of the Ludwig-Maximilians-University of Munich (reference number 18-700).

4.2. Immunohistochemical Peroxidase Staining

Immunohistochemical peroxidase staining was carried out according to the protocol used earlier [50]. After the blocking of endogenous peroxidase and heat pretreatment with a sodium-citrate-buffer (pH 6.0), an incubation with a blocking solution (ZytoChem Plus HRP Polymer System, mouse/rabbit; Zytomed Systems, Berlin, Germany) for five minutes, followed to avoid non-specific staining. Afterwards, the incubation with the primary antibody (anti-RxRα (Perseus Proteomics, Tokyo, Japan) or anti-PPARγ (Abcam, Cambridge, UK, antibody validation by manufacturer)) for 16 h at 4 °C in a humid chamber in the refrigerator (dilutions in Table 2) followed.

For visualization, the chromogenic 3,3′-diaminoenzidine (DAB; Dako, Glostrup, Denmark) was used. The staining reaction was stopped after a specific period for each primary antibody (RxRα 1 min, PPARγ 90 s) by adding distilled water. Counterstaining of the tissue was performed with Mayer's acidic haematoxylin followed by dehydration in ascending alcohol series and a final Roticlear (Carl Roth GmBH, Karlsruhe, Germany) treatment, resulting in a brown color for positively stained cells and a blue color for negative cells.

Corresponding positive and negative controls can be found in supplemental data for antibody validation (Figure S5 and S6).

To analyze the slides the light microscope "Immunohistochemistry Type 307–148.001 512 686" by Leitz was used. Representative pictures were taken by IH-Camera 3CCD Colour Video Camera by Fissler. For image acquisition, the software "Discuss Version 4,602,017-#233 (Carl C. Hilgers Technical Office) was used. Time and space resolution data: 760 × 574 pixel.

4.3. Double Immunofluorescence Staining of Tissue Slides

The same primary antibodies and dilutions were used for the double immunofluorescence staining as described above (4.2.) except from PPARγ (LSBio, Seattle, Wahington, USA, dilution 1:500) and CK 7 (Novocastra, Wetzlar, Germany, dilution 1:30). The PPARy antibody was chosen for all immunofluorescence stainings despite the rabbit-host, as there was non-specific staining with several other antibodies which were tried.

The staining procedure differed depending on the hosts of the primary antibodies.

In the case of different hosts of the primary antibodies, the preparation of the tissue sections for immunofluorescence staining was performed similarly to immunohistochemical peroxidase staining. For detailed protocol see reference [50]. Secondary antibody was incubated for 30 min at RT (Cy3-labeled goat-anti-rabbit IgG—dilution 1:500 and Alexa Fluor 488-labeled goat-anti-mouse IgG—dilution 1:100) in Dako-dilution medium. After a final wash, the sections were covered with mounting medium containing 4′,6-Diamino-2-phenylindile (DAPI) as nucleic staining.

For the immunofluorescence double staining of PPARγ and the histone modifications, the hosts of the primary antibodies were the same, therefore the staining procedure had to be modified. After pretreatment of the samples similar to the immunohistochemical peroxidase staining, a first serum blocking was performed with 10%-goat serum for 45 min. Afterwards, an incubation with the first primary antibody (PPARγ) for one hour at room temperature followed. After a wash with PBS, the incubation with the first secondary antibody (Cy3-labeled goat-anti-rabbit IgG—dilution 1:500) for 30 min at room temperature followed. From this step on, it was necessary to work in the dark to avoid interference with the light-sensitive secondary antibodies. The next step included the second serum blocking, where normal 10%-rabbit serum was added for 45 min, to block the free binding sites of the anti-rabbit immunoglobulin and therefore to prevent unspecific staining. Afterwards, the sections were rinsed in PBS and the incubation with the second primary antibody (the respective histone-antibody) for one hour as well as incubation with the second secondary antibody (Alexa Fluor 488-labeled

goat-anti-mouse IgG —dilution 1:100) for 30 min at room temperature, followed. After a final wash, the sections were covered with mounting medium containing 4',6-Diamino-2-phenylindile (DAPI) as nucleic staining.

As a negative control for the staining, a specific IgG antibody was used in accordance with the utilized antibodies (Figure S7).

Pictures of the immunofluorescence staining were taken with Zeiss Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany). For analysis the lens "40× CP-Achromat 40×/0.65, Infinity/0.17. Zeiss Part #44 09 50" was used. Dichroic filter cube sets from Omega for DAPI (UV Excitation, blue emission filter: Omega 365BP50; dichroic mirror: Omega 400DCLP, emission (barrier) filter: Omega 465DF60), FITC (Spectra: blue excitation, green emission, Omega cube set: XF100-2 with the following characteristics: excitation filter: 475/40, dichroic mirror: 505DRLP, emissions Filter: 535/45) and TRITC (Spectra: Green excitation, Red emissions; Omega dichroic filter cube: exciter: 525AF45, dichroic mirror: 560DRLP, emission (barrier) filter: 595AF60) were used.

The software AxioVision 4.8.1. was used to analyze the immune fluorescence staining. Image bit depth: 24 mm; time and space resolution data: 760 × 574 pixel. For the evaluation of the immunofluorescence staining intensity analysis of ZEN Software (Zeiss, Oberkochen, Germany) was used.

Table 2. Antibodies used in the immunohistochemistry (IH), immunofluorescence (IF) and immunocytochemistry (IC).

Antibody	Species Isotype	Manufacturer	Dilution	
H3K9ac	Rabbit IgG monoclonal (Clone: Y28)	Abcam	1:200	IH, IF, IC
H3K4me3	Rabbit IgG polyclonal	Abcam	1:100	IH, IF, IC
RxRα	Mouse IgG2a monoclonal (Clone: K8508)	Perseus Proteomics	1:200	IH, IF, IC
PPARγ	Rabbit IgG1 polyclonal	Abcam	1:100	IH
PPARγ	Mouse IgG1 monoclonal (Clone: 8D1H8F4)	Abnova	1:500	IC
PPARγ	Rabbit IgG polyclonal	LSBio	1:500	IF
CK 7	Mouse IgG1 monoclonal (Clone: NCL-L-CK7-OVTL)	Novocastra	1:30	IF
Cy2	Goat-Anti-Mouse IgG	Dianova	1:100	IF, IC
Cy3	Goat-Anti-Rabbit IgG	Dianova	1:500	IF, IC

4.4. Staining Evaluation

The evaluation of the immunohistochemical peroxidase staining was performed by using the semi-quantitative Immunoreactive Score (IRS). In each case, the entire slide was evaluated, and the IRS was formed for syncytium and EVT individually. To calculate the IRS the staining intensity of the specific tissue (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) was multiplied by the percentage of positively stained cells (0 = no staining, 1 = <10% of cells, 2 = 11–50% of cells, 3 = 51–80% of cells, 4 = >80% of cells stained) of the respective tissue. When forming the IRS, the quantification of the staining intensity is graded individually in each case according to the general staining intensity of the stain and is, therefore, a semi-quantitative evaluation. The evaluation of the staining was performed by two independent examiners.

4.5. Isolation of EVT Cells from Placenta Tissue

For the isolation of fresh EVT cells, placentas from healthy mothers were used directly after delivery. At first, the placenta was cut carefully, and the maternal part (decidua basalis) was separated from the fetal side (villous tissue). From here on, the maternal tissue was treated separately and washed several times with cold 0.9% NaCl (Carl Roth, Karlsruhe, Germany). After removing as much blood as possible, the last wash was

performed with cold HBSS (Life Technologies, Carlsbad, USA). The tissue was cut into small pieces and transferred in a glass bottle for digestion.

HBSS-HEPES-buffer was made from 25 mM HEPES (Sigma-Adrich, St. Louis, USA) and HBSS (Life Technologies, Carlsbad, USA) and the pH value was adjusted to 7.4. The DMEM (Sigma-Aldrich, St. Louis, USA) was supplemented with 10% FBS superior (Sigma-Aldrich, St. Louis, USA) and 1% antibiotic-antimycotic 100× (Life Technologies, Carlsbad, USA). For the FACS staining buffer, 0.5% albumin (Carl Roth, Karlsruhe, Germany) and 2 mM EDTA (Sigma Aldrich, St. Louis, USA) were dissolved in D-PBS (Life Technologies, Carlsbad, USA).

Two digestion steps were performed to isolate EVTs. The first step was performed with 0.72 mg/mL trypsin (Sigma Aldrich, St. Louis USA) and 0.02 mg/mL DNAse1 (Roche, Basel, Switzerland) in HBSS-HEPES-buffer for 20 min at 37 °C. The second step was executed with 1 mg/mL Collagenase A (Roche, Basel, Switzerland) and 0.02 mg/mL DNAse1 in DMEM. After pulse centrifugation, the supernatant was layered on a Percoll (cytiva, Washington, USA) gradient. For the preparation of the Percoll-tubes, 20%, 30%, 40%, 50%, 60%, and 70% Percoll-dilutions were set up with HBSS-HEPES-buffer. Afterwards the upper yellowish layer was removed and cells were washed twice with FACS-staining buffer. Lastly, the cells were counted with methylene blue (Stemcell Technologies, Vancouver, Canada) in a Neubauer counting chamber, before being stored on ice.

4.6. FACS of Isolated EVT Cells from Placenta Tissue

To confirm the purity of primarily isolated EVT cells FACS analysis was performed. For intracellular trophoblast-specific CK 7 staining, permeabilization-buffer was made from 0.1% saponin (Carl Roth, Karlsruhe, Germany), 5% FBS superior (Sigma-Aldrich, St. Louis, USA), and D-PBS (Life Technologies, Carlsbad, USA). The Fc receptors were blocked in 10% human serum in D-PBS for 10 min at RT. For the surface staining, the cells were incubated in FACS-staining-buffer with CD45-FITC (BioLegend, San Diego, USA) for 15 min at 4 °C. Then the cells were stained with fixable live/dead dye eFluor780 (Thermofisher, Waltham, USA). Next, they were fixed with 1% PFA for 10 min at RT. For intracellular blocking, the cells were resuspended in permeabilization-buffer and 10% human sera was added for 10 min at 4 °C. Intracellular staining was executed with a CK 7-PE antibody (Abcam, Cambridge, UK) diluted in permeabilization-buffer. Before acquisition, the cells were washed in permeabilization-buffer and FACS-staining-buffer. Flow cytometry was performed on BD FACSCanto II and analyzed with FlowJo version 10.

4.7. Cell Culture of HVT-Cell Line and Primarily Isolated EVT Cells with PPARγ-Agonist and -Antagonist

To determine the influenceability of the histone modifications H3K4me3 and H3K9ac by PPARy, 50,000 HVT respectively primarily isolated EVT cells were seeded in 500 µL medium (RPMI-1640 + 10% FCS) per chamber of a chamber slide. After growing and adhesion to the slides, the cells were treated with the PPARγ-agonist Ciglitazone (20 mM, Tocris Bioscience, Bristol, UK) [69,70,76] and the PPARγ antagonist T0070907 (50 mM, Tocris Bioscience, Bristol, UK) or respective control vehicle. After an incubation period of 24 h, immunofluorescence staining was performed. The concentrations of the PPARγ-agonist Ciglitazone and the PPARγ antagonist T0070907, as well as the incubation period were chosen according to the literature published with these chemicals [69–71,76,77].

4.8. Double Immunofluorescence Staining of Chamber Slides

Fixation of the incubated cells in the chamber slides was carried out for 10 min in an ice-cold mixture of 100% methanol and ethanol in a relation of 1:1. After air-drying the slides, blocking, and staining of the primary antibody with combinations of H3K4me3, H3K9ac, and PPARγ (for dilutions see Table 1) was performed as described for the double immunofluorescence staining of tissue slides. Since no unspecific staining could be

determined within the HVT-cells respectively isolated EVT, a PPARγ antibody with a mouse host was used (Abnova, Taipeh, Taiwan—dilution 1:100).

The slides were washed with PBS between all individual steps. Secondary antibody staining and covering was performed as for the double immunofluorescence staining of tissue slides. Corresponding negative controls can be found in the supplement (Figure S7). Representative pictures for analysis were taken with a confocal laser scan microscope (LSM 510 Meta, Zeiss). LSM 510 Meta 18 confocal laser scanning microscope consists of an Axiovert 200M equipped with Differential Interference Contrast (DIC) and with a range of excitation laser lines: Ar diode laser: 405 nm (30 mW), Ar: 458, 477, 488, 514 nm (30 mW), HeNe: 543 nm (1 mW), HeNe: 633 nm (5 mW). Used lens for analysis: 63× Plan-Apochromat NA 1.4, d in mm 0.19 from Zeiss.

4.9. Quantitative Analysis of Double Immunofluorescence Staining

For quantitative analysis of the staining intensity of the immunofluorescence staining of cultured trophoblasts, the ZEN Software (Zeiss, Oberkochen, Germany) was used. The mean fluorescence intensity of the investigated histone, receptor or protein was measured in three visual fields. The intensity of the concerned channel was evaluated per area (μm^2) or set in relation to the DAPI channel to exclude the confounder of cell number in each visual field. The average of all visual fields was calculated and plotted in a diagram ± SEM.

4.10. Statistical Analysis

Statistical analysis was performed using SPSS (version 26 IBM company, Chicago, IL) and GraphPad Prism (Version 6.0 GraphPad Software, La Jolla, CA). Non-parametric tests were used for statistical analysis, due to not normally distributed data. Mann-Whitney-U-test was chosen for independent samples and Wilcoxon-signed-rank-test for paired samples. Results of these tests are given as mean value ± SD. For correlation analysis, Spearman–Rho correlation test was used. The correlation coefficient r indicates the strength of the correlation ($r < 0.3$ weak relation, $r > 0.3$ medium relation, $r > 0.5$ strong relation) [56]. For the double immunofluorescence staining of cultured trophoblasts, the t-test was used to examine differences between the two groups. The significance level for all tests was assumed at $p < 0.05$.

5. Conclusion

In summary, we showed that the expression of PPARγ regulates H3K4me3 and H3K9ac in HVT cells and primary isolated healthy EVT cells in a positive manner. Our in vivo results indicate that a reduced PPARγ expression correlates with the analyzed histone modifications and that an increased PPARγ activity might inhibit H3K4me3 and H3K9ac during PE. If considering our findings in relation to results from other studies, one can assume that a decreased expression of PPARγ is accompanied by increased activity. Thus, activation of PPARγ leads to a downregulation of the investigated histone modifications.

Whether H3K4me3 and H3K9ac act only as indicators for an abnormal trophoblast invasion through a lack of PPARγ-expression in PE, or whether they are responsible for defective placentation cannot be concluded from our results.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/article/10.3390/ijms222212469/s1.

Author Contributions: Conceptualization. U.J. and S.M. (Sarah Meister); methodology, S.M. (Sarah Meister) and K.S.; software, S.B.; validation, C.K., V.v.S.; formal analysis, C.S.; investigation, L.H., C.P., S.M. (Sophie Mitter); data curation, L.H.; writing—original draft preparation, S.M. (Sarah Meister); writing—data interpretation and editing: S.M. (Sarah Meister), L.H., S.B., C.P, S.M. (Sophie Mitter), C.K., V.v.S., S.C., K.S., C.S., T.M.K., S.M. (Sven Mahner), U.J. and T.K.; visualization, C.K.; supervision, U.J.; project administration, T.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The present study was approved by the local ethics committee of the Ludwig-Maximilians-University of Munich (reference number 18-700). The translated statement: The ethics committee can grant your study the ethical-legal harmlessness.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: S.M (Sven Mahner): Research support, advisory board, honoraria and travel expenses from AbbVie, AstraZeneca, Clovis, Eisai, GlaxoSmithKline, Medac, MSD, Novartis, Olympus, PharmaMar, Roche, Sensor Kinesis, Teva, Tesaro; TK: holds stock of Roche, relative employed at Roche; TMK: holds stock of Roche, employed at Roche.

Abbreviations

CK 7	cytokeratin 7
CK 7-PE	phycoerythrin labeled cytokeratin 7
Cy2	cyanine dyes 2 for fluorescence staining
Cy3	cyanine dyes 3 for fluorescence staining
D-PBS	dulbecco's phosphate buffered saline
DAB	chromogenic 3,3′-diaminoenzidine
DAPI	4′,6-Diamino-2-phenylindile
DMEM	dulbecco's Modified Eagle's Medium
EDTA	ethylenediaminetetraacetic acid
EVT	extravillous trophoblast
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
H3K4me3	trimethylated lysine of the histone H3
H3K9ac	acetylated lysine of the histone H3
HBSS	Hanks' balanced salt solution Buffer
HBSS-HEPES-buffer	Hanks' balanced salt solution Buffer with 10mM Hepes
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
HVT	human villous trophoblasts, a cell line of cytotrophoblasts
IRS	Immunoreactive Score
IUGR	intrauterine growth retardation
LMU	Ludwig Maximilian University of Munich
NaCl	sodium chloride
PBS	phosphate-buffered saline
PE	preeclampsia (respectively: preeclampsia placentas)
Pen	penicillin
PFA	perfluoroalkoxy alkane
PG	prostaglandins
PPARγ	peroxisome proliferator-activated receptor gamma
RPMI-1640	Roswell Park Memorial Institute 1640
RT	room temperature
RUPP	reduced uterine perfusion pressure

RxRα	retinoid X receptor alpha
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
Strep	streptomycin
T0070907	antagonist of PPARγ
VEGF	vascular endothelial growth factor

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VI. Paper III

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Article

Regulatory T Cell Apoptosis during Preeclampsia May Be Prevented by Gal-2

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Abstract: There are several open questions to be answered regarding the pathophysiology of the development of preeclampsia (PE). Numerous factors are involved in its genesis, such as defective placentation, vascular impairment, and an altered immune response. The activation of the adaptive and innate immune system represents an immunologic, particularity during PE. Proinflammatory cytokines are predominantly produced, whereas immune regulatory and immune suppressive factors are diminished in PE. In the present study, we focused on the recruitment of regulatory T cells (Tregs) which are key players in processes mediating immune tolerance. To identify Tregs in the decidua, an immunohistochemical staining of FoxP3 of 32 PE and 34 control placentas was performed. A clearly reduced number of FoxP3-positive cells in the decidua of preeclamptic women could be shown in our analysis (p = 0.036). Furthermore, CCL22, a well-known Treg chemoattractant, was immunohistochemically evaluated. Interestingly, CCL22 expression was increased at the maternal-fetal interface in PE-affected pregnancies ($p_{syncytiotrophoblast}$ = 0.035, $p_{decidua}$ = 0.004). Therefore, the hypothesis that Tregs undergo apoptosis at the materno-fetal interface during PE was generated, and verified by FoxP3/TUNEL (TdT-mediated dUTP-biotin nick end labeling) staining. Galectin-2 (Gal-2), a member of the family of carbohydrate-binding proteins, which is known to be downregulated during PE, seems to play a pivotal role in T cell apoptosis. By performing a cell culture experiment with isolated Tregs, we could identify Gal-2 as a factor that seems to prevent the apoptosis of Tregs. Our findings point to a cascade of apoptosis of Tregs at the materno-fetal interface during PE. Gal-2 might be a potential therapeutic target in PE to regulate immune tolerance.

Keywords: regulatory T cells; apoptosis; preeclampsia; Galectin-2

1. Introduction

Hypertensive disorders are a common complication in pregnancy, resulting in an increased risk of further complications, as well as long-term consequences for women and their fetuses [1–3]. Preeclampsia (PE), one of these hypertensive disorders, represents a severe pregnancy complication affecting 2–5% of all pregnancies [4]. The main diagnostic criteria of PE are based on the following symptoms: a new onset hypertonia (>140/90 mmHg), combined with proteinuria (>300 mg/24 h) or other organ dysfunction in the second half of pregnancy [5]. PE is associated with high morbidity and mortality, causing 70,000 maternal deaths worldwide per year [6].

Due to the lack of a cause-specific therapy for PE [6,7], details about the pathophysiology of this mechanism need to be further elucidated. However, the development of PE cannot be attributed to one particular cause, since numerous factors are involved in the pathophysiology. Currently, it is known that the pathogenesis of PE progresses in two stages, beginning with a defective trophoblast invasion and spiral artery remodeling, as well as immunological alterations in the early materno-fetal environment. Later in pregnancy, the reduced uteroplacental flow promotes the release of proinflammatory chemokines, inducing a systemic inflammation [8]. Overall, the combination of inflammatory processes [4,9–11], the loss of the maternal tolerance towards the fetus [3,12,13], and a maternal cardiovascular maladaptation [14,15] are important elements. Further, an impaired trophoblast invasion [16,17]—resulting, among other complications, as a general vascular dysfunction and a deficient remodeling of the spiral arteries [5,18,19]—as well as defective placentation [15,20–22] appear to lead to placental insufficiency [23] and the release of vasoactive and pro-inflammatory substances that seem to cause the clinical symptoms.

Different studies underline the importance of the maternal immune system in the pathophysiology of PE, assuming an inadequate immune tolerance towards the semi-allogenic fetus that leads to the abnormal trophoblast invasion [24–26]. Furthermore, a shifted cytokine secretion of activated T cells towards the Th1 profile, stimulating a proinflammatory function, has already been shown in PE [27]. Other studies detected an extensive activation of either circulating or decidual neutrophils and monocytes in PE [28,29]. In addition, regulatory T cells (Tregs) have been identified as key players in several processes mediating immune tolerance, as in organ transplantation [30]. They are, moreover, assumed as an important immune cell population for the maintenance of the materno-fetal tolerance via the inhibition of natural killer cells (NK), natural killer T cells (NK-T), and T-lymphocytes [31,32].

The amount of circulating and resident Tregs increases in healthy pregnancies until the end of the 2nd trimester [33]. However, in the case of PE, several studies detected a decrease of Tregs in the maternal peripheral blood during pregnancy [34,35], whereas there is hardly any data about decidual Treg recruitment during PE, and existing data are inconsistent. One study has already shown, that Tregs were less located in the decidua of mice [36]. The mechanism of Treg recruitment in PE has not been fully elucidated. However, in other diseases, such as carcinoma [37,38], auto immune diseases [39], infections [40,41], or implantation [42,43], CCL22, the macrophage derived chemokine, and its receptor, CCR4, are well known for their role in the migration of Tregs [44]. CCL22 is produced by certain types of immune cells, such as macrophages, monocyte-derived dendritic cells [45,46], NK cells, and activated T cells [47].

Gal-2, is a member of the family of carbohydrate-binding proteins that participate in multiple cellular mechanisms, such as cell adhesion and activation, cytokine secretion, immune cell migration, and apoptosis, by binding distinct cell surface or extracellular matrix glycoconjugates [48–51]. Furthermore, galectins are associated with cell death and growth, as well as with cell differentiation, in addition to their modulatory effect on the immune system by regulating monocytes, macrophages, and $CD8^+$-T cells. Gal-2 is able to bind to T cells in a β-galactoside-specific manner to induce apoptosis in activated T cells [52–54]. Structurally, Gal-2 is closely related to galectin-1 (Gal-1), although it acts via

different cell surface binding strategies [55]. While little is known about the relationship between Gal-2 and Tregs, Gal-1 is considered as a negative regulator of the immune response promoting Treg induction, differentiation, and expansion [56,57]. In PE and other pregnancy diseases, the Gal-2 expression is downregulated in the placental tissue [58], in contrast to the increased Gal-2 level in maternal blood during preeclampsia [59]. Still, there is a lack of research regarding the role of Gal-2 during pregnancy affected by PE.

Therefore, the present study targeted the investigation of Tregs' recruitment in the decidua of PE-affected pregnancies, as well as their behavioral alterations concerning apoptosis and their chemokine attractant CCL22. Furthermore, the effect of Gal-2 on Tregs in cell culture undergoing apoptosis was observed.

2. Results

2.1. The Number of Tregs Is Decreased in the Decidua of PE-affected Pregnancies

The number of Tregs was evaluated by immunohistochemical FoxP3 staining. Cells were counted in three randomly selected visual fields of the decidua and the average was calculated.

The number of FoxP3-positive cells was significantly reduced (p = 0.046) in PE placentas (1.07 ± 1.203 and a range from 0 to 3.67) compared to control placentas (1.80 ± 1.497 and a range from 0 to 6.33) (Figure 1 A–C).

In addition, since the weeks of gestation of the observed PE placentas vary over a wide range, the number of Tregs in early-onset PE (before the 34th week of gestation) and late-onset PE (after the 34th week of gestation) was compared. Although there was a descriptive difference between early-onset PE (1.67 ± 1.553) and late-onset PE (0.84 ± 0.992), this difference was not significant (p = 0.218).

Considering only the control placentas, a significantly positive correlation of the number of Tregs and maternal age at birth (r = 0.550, p = 0.001) was found.

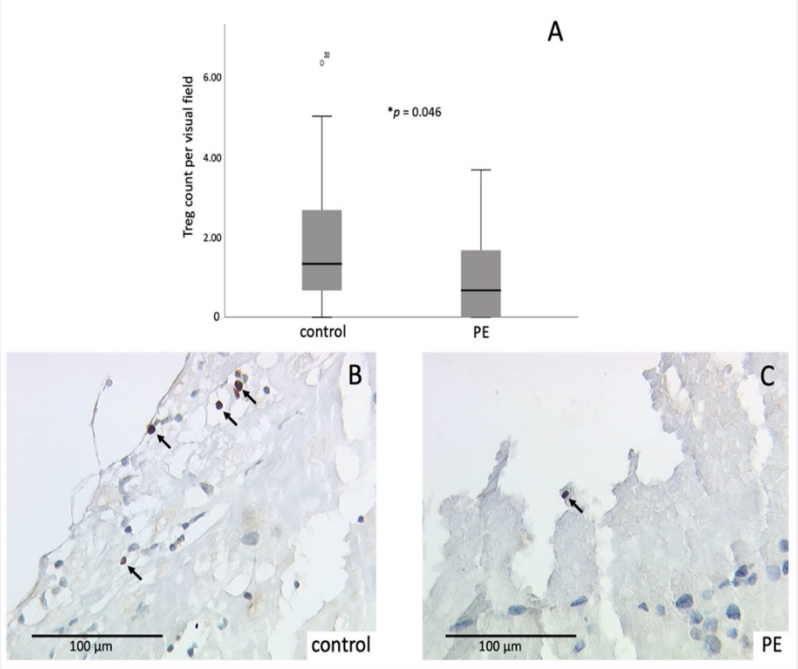

Figure 1. Immunohistochemical staining results of FoxP3: (**A**) boxplot of the average number of FoxP3-positive Tregs per visual field in control and PE placentas (control n = 34, PE n= 30), mean ±

SD; *p*-values were calculated with Mann-Whitney-U-Test, **p* = 0.046; (**B**) representative picture of control placenta; (**C**) representative picture of PE placenta. Detected Tregs are marked with arrows. The circle in **A** symbolizes an outlier value with its respective number for identification. A respective negative and positive control is shown in the Supplementary Data (Figure S1).

2.2. CCL22 Expression Is Increased in PE Compared to Control Placentas

The expression of CCL22, which is known to be involved in Treg recruitment, was evaluated individually for the different tissue parts of the placenta. The staining result of the syncytium, evaluated by the mean IRS (International Remmele Score), showed a significantly increased CCL22 cytoplasmatic expression in the PE placentas (4.00 ± 3.006) compared to the control placentas (2.38 ± 1.688; $p = 0.013$) (Figure 2). Further, an analysis of the mean intensity of the CCL22 staining was performed. There, a significantly increased intensity was measured in PE placentas (0.172 ± 0.0079) compared to the controls (0.161 ± 0.0151; $p < 0.001$).

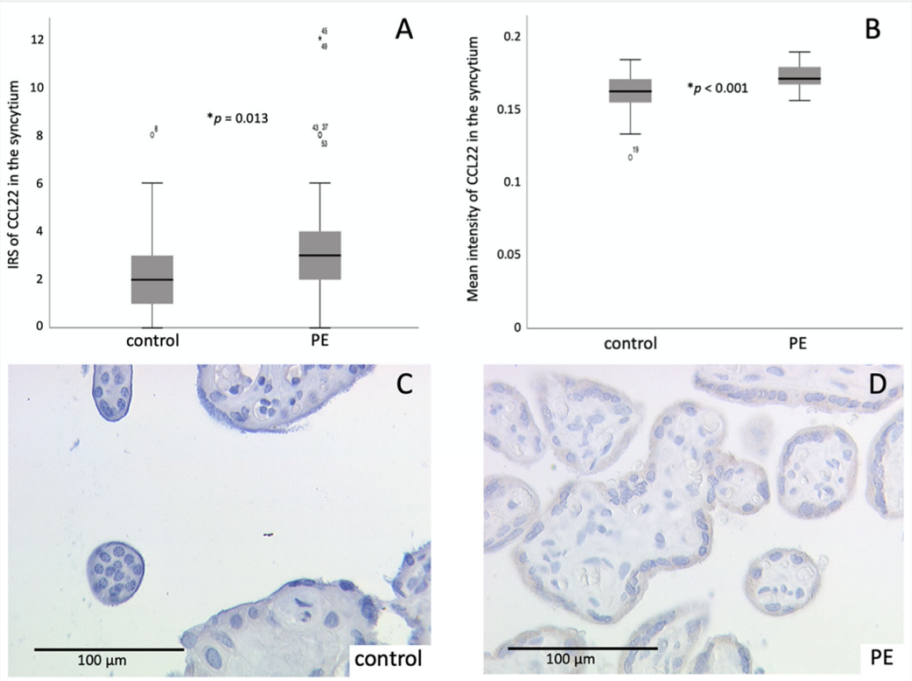

Figure 2. Immunohistochemichal staining results of CCL22 in the syncytium: (**A**) boxplot of the mean IRS of syncytial staining of CCL22 in control and PE placentas (control $n = 34$, PE $n = 30$), mean ± SD; *p*-values were calculated with Mann-Whitney-U-Test, **p* = 0.013; (**B**) boxplot of the mean intensity of syncytial staining of CCL22 in control and PE placentas (control $n = 34$, PE $n = 30$), mean ± SD; *p*-values were calculated with Mann-Whitney-U-Test, **p* < 0.001; (**C**); representative picture of control placenta (IRS = 3); (**D**) representative picture of PE placenta (IRS = 12). The circles in **A** and **B** symbolize outlier values with their respective number for identification. The star in **A** symbolizes an extreme outlier value with its respective number for identification. A respective negative and positive control is shown in the Supplementary Data (Figure S2).

Furthermore, the mean IRS of the CCL22 staining in the decidual part of the placenta was significantly higher (p = 0.006) during PE (2.30 ± 2.693) compared to healthy samples (0.76 ± 1.130) (Figure 3). The mean-intensity-analysis did not show a significant difference

between PE and the control group. However, a higher mean intensity of CCL22 was detected in the PE group (0.166 ± 0.0125) compared to the control group (0.161 ± 0.0153).

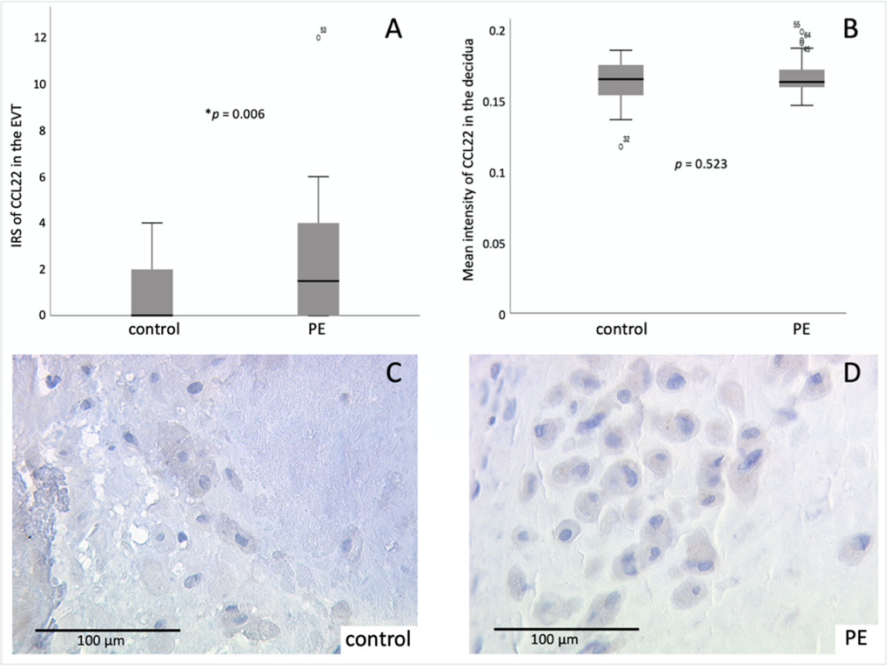

Figure 3. Immunohistochemical staining results of CCL22 in the EVT (extravillous trophoblast): (**A**) boxplot of the mean IRS of decidual staining of CCL22 in control and PE placentas (control n = 34, PE n= 30), mean ± SD; p-values were calculated with Mann-Whitney-U-Test; (**B**) boxplot of the mean intensity of decidual staining of CCL22 in control and PE placentas (control n = 34, PE n= 30), mean ± SD; p-values were calculated with Mann-Whitney-U-Test; (**C**) representative picture of control placenta (IRS = 3), (**D**) representative picture of PE placenta (IRS = 6). The circles in (**A**) and (**B**) symbolize outlier values. Respective negative and positive control is shown in the Supplementary Data (Figure S2).

Moreover, the expression of CCL22 in the syncytiotrophoblast and in the decidua correlated significantly positive (r_{IRS} = 0.401, p_{IRS} = 0.001; $r_{intensity}$ = 0.442, $p_{intensity}$ < 0.001), indicating an increased expression in the entire placenta in the case of PE.

In addition, since the weeks of gestation of PE placentas vary over a wide range, the expression of CCL22 in early-onset PE and late-onset PE was compared. Even though there was a descriptive difference between early-onset PE ($IRS_{syncytium}$ = 5.88 ± 4.390, IRS_{EVT} = 2.63 ± 2.504; $intensity_{syncytium}$ = 0.174 ± 0.1016, $intensity_{decidua}$ = 0.170 ± 0.1506) and late-onset PE ($IRS_{syncytium}$ = 3.32 ± 2.056, IRS_{EVT} = 2.18 ± 2.805; $intensity_{syncytium}$ = 0.171 ± 0.0070, $intensity_{decidua}$ = 0.165 ± 0.0116), this difference emerged as insignificant (IRS: $p_{syncytium}$ = 0.277, p_{EVT} = 0.534; intensity: $p_{syncytium}$ = 0.730, $p_{decidua}$ = 0.219).

2.3. Identification of Decidual Cells Expressing CCL22 as EVT

To investigate the type of decidual cells expressing CCL22, an immunofluorescence staining of CCL22 and CK7 was performed. This staining showed a coexpression of CCL22 and CK7 in all cells stained by anti-CCL22 antibody. Thus, the CCL22-expressing cells in the decidua can be clearly classified as EVT, since there are hardly any other

trophoblasts in third trimester placentas and CK7 accounts as a specific trophoblast marker [60]. The result for the double expression in the control and PE placentas was nearly identical (Figure 4).

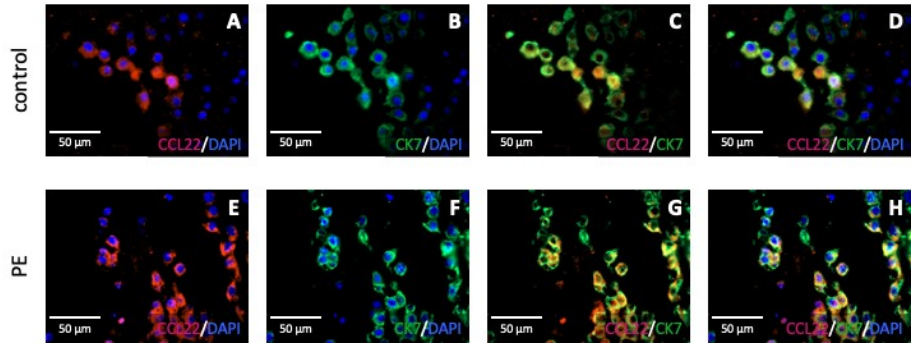

Figure 4. Immunofluorescence staining of CCL22 (red) and CK7 (green) in the decidua of PE and control placentas. Nuclear staining with DAPI is shown in blue in each case. Representative pictures of control placenta (**A**–**D**) and PE placenta (**E**–**H**), single staining of CCL22 (**A**,**E**) and CK7 (**B**,**F**), double staining of CCL22 and CK7 (**C**,**G**), and merge including nuclear staining (**D**,**H**) are shown. Respective negative control picture is shown in the Supplementary Data (Figure S3).

2.4. CCL22 and FoxP3 Are Correlating Positively

The expression of CCL22 and the number of placental Tregs correlated significantly positive in the EVT (r = 0.264, p = 0.038; Figure S4) but not the syncytiotrophoblast (r = 0.239, p = 0.061; Figure S5). Individual examination of PE and controls revealed a significantly positive correlation between the number of Tregs and the expression of CCL22 in the syncytium in PE placentas (r = 0.576, p = 0.001) and a significantly positive correlation of the number of Tregs and the expression of CCL22 in the EVT regarding the control placentas (r = 0.465, p = 0.006).

2.5. Tregs Undergo Apoptosis in PE

To understand the decreased number of FoxP3-positive cells in PE placentas despite increased CLL22 expression, TUNEL staining was performed to identify apoptotic Tregs. A clear difference between the control group and the PE group in the percentage of TUNEL-positive Tregs was found. While only 20–30% apoptotic Tregs appeared in the control group, almost 100% of the detected Tregs were undergoing apoptosis in the PE group (Figure 5).

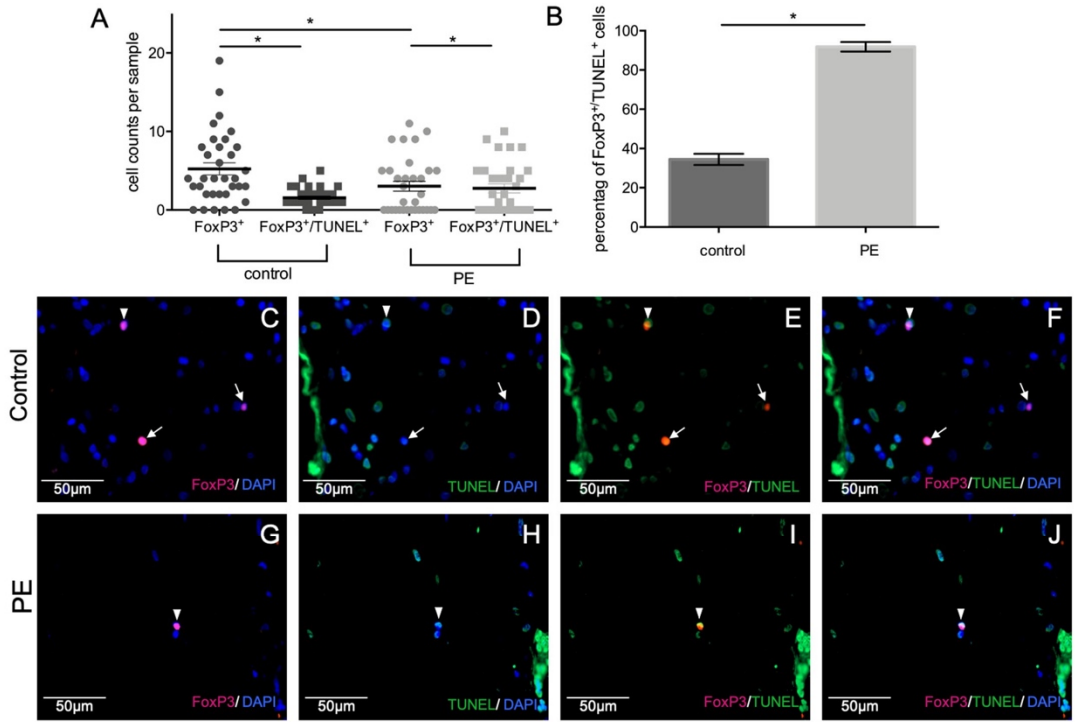

Figure 5. Immunofluorescence staining of apoptotic Tregs. Graphical representation of all Tregs ($FoxP3^+$) and all apoptotic Tregs ($FoxP3^+/TUNEL^+$) counted per sample (**A**), mean ± SD; p-values were calculated with Mann-Whitney-U-Test; mean percentage of apoptotic Tregs counted in controls and PE (**B**), mean ± SD; *p*-values were calculated with Mann-Whitney-U-Test; Representative pictures of control placenta (**C**–**F**) and PE placenta (**G**–**J**), single staining of FoxP3-positive Tregs (red) (**C**,**G**), single TUNEL staining (green) (**D**,**H**), double staining of apopototic Tregs (**E**,**I**) (yellow), merge (**F**,**J**), and nuclear staining with DAPI (**C**,**D**,**F**,**G**,**H**,**J**). Non-apoptotic Tregs are marked with arrows; apoptotic Tregs are marked with arrowheads. A respective negative control is shown in the Supplementary Data (Figure S3).

2.6. Correlation of Gal-2 with Tregs

Since galectins are known to be able to induce or inhibit the apoptosis of T cells [55,61,62], data about the expression of galectins—which was detected by our group earlier and published by Hutter et al. [53]—were correlated with the number of Tregs (Figures S6 and S7). A significant positive correlation between Gal-2 in the syncytiotrophoblast and the number of decidual Tregs was detected ($r = 0.390$, $p = 0.049$). Considering the control and PE placentas individually, a significantly positive correlation was shown between the number of Tregs and the expression of Gal-2 in PE placentas (Gal-2 in the syncytium: $r = 0.620$, $p = 0.042$; Gal-2 in the decidua: $r = 0.720$, $p = 0.012$).

2.7. Gal 2 Protects Tregs from Apoptosis

Since a positive correlation between the number of Tregs and Gal-2 expression, as well as an increased ratio of apoptotic Tregs, was detected in PE placentas, the influence of Gal-2 on the apoptosis of Tregs was investigated. Therefore, the apoptosis in Tregs isolated from blood of healthy patients was induced by FAS ligand (FasL) with and without the addition of Gal-2 and compared with an untreated Treg control group.

Since the results varied highly between the donors, the measured levels of Caspase 3 were set in relation to the Treg + FasL group donor-specifically, further termed as standardized values. The Treg + FasL group was chosen as the reference group, since the highest apoptotic rate was suspected in this group.

Our results showed a significantly reduced amount of active Caspase 3, which is an indicator of active apoptosis, in Tregs incubated with Gal-2 and FasL compared to the group incubated with solely FasL ($p_{standardized}$ = 0.001, $p_{concentration}$ = 0.161).

As one donor showed a higher rate of apoptosis in the untreated Tregs than in those treated with FasL, that sample was excluded from the overall statistical analysis, as it can be assumed that the apoptosis induction was defective. Nevertheless, a descriptive analysis showed a reduction in the level of Caspase 3 after addition of Gal-2 in this donor as well (Treg + FasL = 45.87 ± 19.45; Treg + FasL + Gal-2 = 16.99 ± 22.71).

The concentration of active Caspase 3 showed significant differences between the untreated Treg group, the Treg + FasL group, and the Treg + FasL + Gal-2 group (Treg = 81.56 ± 91.979, Treg + FasL = 437.02 ± 43.915, Treg + FasL + Gal-2 = 260.39 ± 147.971; p = 0.004). The pairwise comparison of the single groups revealed significant differences between the groups Treg and Treg + FasL (p = 0.001) but not between the groups Treg + FasL and Treg + FasL + Gal-2 (p = 0.054) (Figure S8).

In contrast, standardized values showed an even higher significance between the untreated control group and the FasL group (p = <0.001), as well as significant differences between the groups with and without Gal-2 (p = 0.018) (Figure 6). Therefore, the lack of a significant difference in the measured levels of Caspase 3 seems to occur through donor-specific differences.

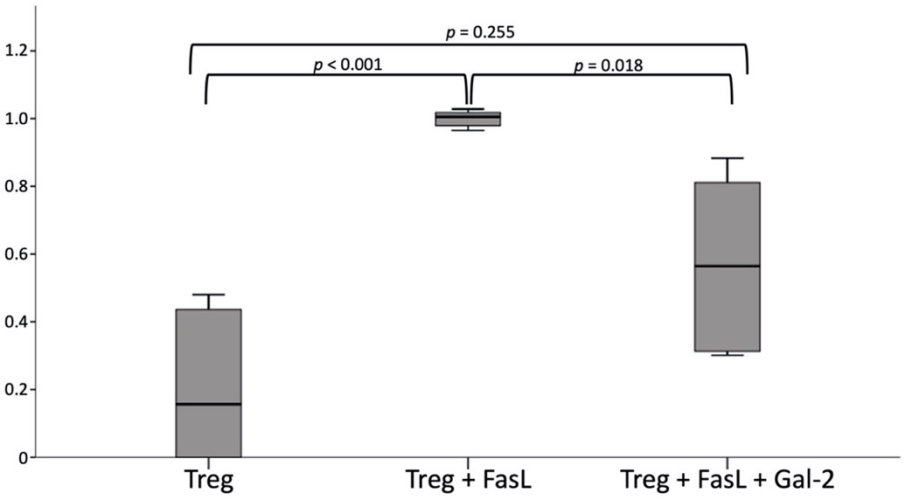

Figure 6. Concentration of standardized active Caspase 3 after exclusion of the second donor, in the groups untreated Treg (Treg, 0.20 ± 0.231), Treg with induction of apoptosis through FasL (Treg + FasL, 1.00 ± 0.024), and the group with the Gal-2 treatment (Treg + FasL + Gal-2, 0.57 ± 0.283). The one-way-ANOVA-Kruskal-Wallis-Test showed significant differences among the three groups (p = 0.002). Further analysis revealed this significant difference between the group Treg and Treg + FasL (p < 0.001), as well as between Treg + FasL and the group Treg+FasL+Gal2 (p = 0.018).

3. Discussion

Various theories for placental dysfunction during the pathophysiology of PE exist: oxidative stress [63,64], generation and transformation of the spiral arteries [65,66], and the imbalance between the maternal adaptive immune system as a proinflammatory

response and a lack of immune tolerance towards the semi-allogenic fetus [24,25]. Therefore, the role of the maternal immune response during the development of PE needs to be further investigated to elucidate pathophysiologic mechanisms of PE and to discover potential therapeutic targets. In the present study, we were able to detect a significantly reduced number of FoxP3positive cells, considered to be Tregs, in the decidua of PE-affected pregnancies despite an upregulation of CCL22, a potent Treg chemoattractant. Furthermore, we detected higher rates of apoptotic Tregs in PE placentas. Gal-2, a well-known immunoregulator, which is downregulated in PE placentas, could be identified to protect Tregs from apoptosis in vitro.

Tregs are known to play an essential role in controlling immune regulatory processes. Since there are incoherent findings about Tregs' recruitment in PE-affected pregnancies and the importance of Tregs during implantation had already been demonstrated in mice [67], the aim of the present investigation was to clarify the aspect of decidual Tregs' recruitment and to elucidate their role during PE. The present study detected a reduced number of FoxP3-positive cells in the decidua of PE placentas, supporting the findings from previous research that showed reduced levels of circulating and decidual resident Tregs [67,68] in pregnancies suffering from PE.

However, when interpreting the results, the differing level of Tregs during pregnancy needs to be considered when interpreting our results. Overall, the number of placental Tregs peaks in the second trimester, followed by decreasing values towards the end of pregnancy [33,69,70]. Since the different weeks of gestation in the PE and control groups could be excluded as a cause for the different number of Tregs through regression analysis and pregnancy week matched analysis (which may be seen in the Supplementary Data), we aimed to analyze the recruitment of Tregs as a possible explanation for the lower number of decidual Tregs in PE, assuming that lower chemoattractant levels might be responsible for the reduced Treg infiltration. Therefore, we chose to analyze CCL22, a well-known chemoattractant for Tregs [71].

Generally, the role of CCL22 during pregnancy is not completely resolved and CCL22 expression in the placental tissue has not been previously investigated during preeclampsia. Still, CCL22 is known to be expressed by dendritic cells and macrophages, both of which account for a large proportion of decidual immune cells [72], as well as in trophoblasts and maternal stromal cells [42]. Macrophages are essential players in remodeling the uterine vasculature, thereby facilitating an adequate placental–fetal blood supply [73,74]. Furthermore, as an immune cell-derived cytokine, CCL22 is involved in M2 polarization of placental macrophages [75]. These immune cells enhance endocytosis and promote tissue repairing mechanisms and cell growth, as well as tissue remodeling. They further promote maternal immune tolerance against the semi-allogenic fetus and preserve fetal growth until delivery [76,77]. Further CCL22 was revealed as a marker for preeclampsia in one study where the serum of pregnant women was analyzed [78]. In accordance with our results, which showed a significantly higher expression of CCL22 in PE placentas, Freier et al. [42] generated the hypothesis of placental CCL22 acting as a negative feedback response to proinflammatory events, since they found no decidual CCL22 expression in healthy first trimester placenta, in contrast to an increased decidual expression in recurrent miscarriages. Since we were able to identify EVT cells expressing CCL22 via immunofluorescence, we could confirm the findings of Freier et al. [42], stating that CCL22 is not only secreted by solid tumor cells, epithelial cells, and immune cells, such as monocytes and macrophages [79–81]. Moreover, this could explain the recruitment of Tregs by trophoblast cells, which was already shown by several studies [82,83]. Until now, hCG was assumed to be one of the potential attractants, whereas downregulation of hCG production after siRNA intervention led to reduced Treg recruitment [84]. Nevertheless, the results of the present study hinted at trophoblasts being able to secrete CCL22. However, future research is needed to support this theory, including further confirmation by in vitro and in vivo investigations.

Although we found a diminished number of decidual Tregs but a significantly higher expression of CCL22 in PE placentas, the amount of decidual Tregs correlated significantly positive with the placental CCL22 level. Therefore, the hypothesis of inhibited Treg recruitment by a lack of CCL22 in preeclamptic placenta could be refuted.

Earlier studies had already shown an impaired function of Tregs during PE [85,86]. Furthermore, Zhang et al. [87] found a reduced proliferation of placental Tregs in preeclampsia by analyzing the Ki67 −/+ Tregs in the placenta. Therefore, we hypothesized that the known impaired trophoblast function in PE, as well as the decreased number of Tregs, seemed to be indicative for an increased apoptosis of decidual Tregs in PE placentas. In this context, we investigated the apoptosis of Tregs via TUNEL staining. By analyzing the co-expression of FoxP3, DAPI, and TUNEL staining, we detected an increased number of TUNEL-positive Tregs in PE compared to control placentas. Therefore, enhanced apoptosis of Tregs in preeclamptic placentas might explain the reduced number of decidual Tregs. Increased Treg apoptosis during PE might be a possible explanation for the reduced number of Tregs in PE placentas despite an increased expression of CCL22, one important Treg chemokine. Nevertheless, there are other chemokine ligands, such as CX3CL1 [88,89] CCL3, CCL4, and CCL5 [90–92], whose receptors are further expressed by Tregs during pregnancy [93], potentially contributing to chemokine-mediated migration to the decidua.

The imbalance of proinflammatory and anti-inflammatory acting T cells has already been described in pregnancies affected by PE [94]. A reduction or impairment of the immune modulating Tregs induced by apoptosis might be responsible for a lack of immune tolerance against the semi-allogenic fetus. Since the inappropriate and proinflammatory activation of the immune system is thought to play a considerable role in the development of PE [78], the prevention of Treg apoptosis might account for a potential therapeutic target of this pregnancy-associated disease. To identify a potential factor that could help to prevent Treg apoptosis, we chose Gal-2 to perform in vitro experiments. Gal-2, a member of the family of carbohydrate-binding proteins, plays a pivotal role in T cell apoptosis [55,61,62]. Since the expression of Gal-2 is known to be decreased in PE placentas [53], we performed a correlation analysis of Gal-2 and the number of decidual Tregs, which showed a significantly positive correlation. To analyze the possible effect of Gal-2 on Tregs undergoing apoptosis, apoptosis was induced in primary isolated Tregs via FasL. We showed a significantly reduced level of active Caspase 3 in Gal-2 co-cultured cells, indicating a clearly protective effect of Gal-2 on Tregs undergoing apoptosis.

Although the present study revealed promising results, the research conducted also had some limitations that need to be discussed. While in past studies different subtypes of Tregs have been identified in pregnancy and PE, our study only focused on FoxP3-positive cells and did not differentiate between, e.g., iTregs (Helios-) and nTregs (Helios+), while Hsu et al. [50] found no significant difference in decidual FoxP3+ Tregs. Furthermore, to confirm the protection from apoptosis through Gal-2, primary isolated Tregs from healthy control patients were used. Since it was shown that decidual Tregs are phenotypically distinct from peripheral blood Tregs, this difference needs to be considered when interpreting our results [95,96]. Therefore, future research is needed, using Tregs from pregnant women's blood or directly from the decidual tissue, to confirm that Gal-2 might be a potential therapeutic target for PE and the protecting effect on apoptosis of Treg apoptosis.

4. Materials and Methods

4.1. Sample Placental Tissue

Tissue samples were collected from the Department of Obstetrics and Gynecology of the University Hospital, LMU Munich, between 2007 and 2019. The collective consisted of 32 PE placentas and 34 control placentas with a mean maternal age of 32.37 ± 5.659 years

(range: 17–44 years). The weeks of gestation at birth differed significantly, with a range between 25 and 40 weeks ($p < 0.001$) between PE and controls; therefore, linear regressions were performed. With regard to both the number of Tregs ($p = 0.339$), and the expression of CCL22 ($p_{syncytium} = 0.064$; $p_{EVT} = 0.350$), no significant impact of the weeks of gestation was detected (Figures S9–S13). Since the weeks of gestation were missing from two PE placentas, whereas Tregs are known to change in number during ongoing pregnancy, we excluded these two PE placentas from the analysis, but separately reported the analysis including the two placentas in the Supplementary Materials. The gender of the newborns was balanced, with 30 female and 31 male newborns (controls: 18 females, 16 males; PE: 12 females, 15 males) (Table S1).

4.2. Immunohistochemistry

The two immunohistochemical stainings were performed according to different protocols. Unless otherwise stated, the work was carried out at room temperature.

4.2.1. FoxP3 Staining

After deparaffinization of the slides in Roticlear (Carlroth, Arlesheim, Switzerland) for 20 min, the endogenous peroxidase was blocked by a 3% H2O2 methanol mixture. Rehydration by descending alcohol series was followed by demasking via heat pretreatment using a sodium citrate buffer (pH = 6.0). To prevent an unspecific binding and staining, an incubation with Blocking Solution Reagent 1 (ZytoChem Plus HRP Polymer System; Zytomed Systems, Berlin, Germany) for five minutes was performed. Subsequently, the primary antibody Anti-FoxP3 (Abcam, Cambridge, UK; mouse IgG monoclonal, Clone: 236A/E7; dilution 1:300) was applied to incubate for 16 h at 4 °C. After incubation with the primary antibody, a 20 min incubation with Post-Block Reagent 2 (ZytoChem Plus HRP Polymer System; Zytomed Systems, Berlin, Germany) and a 30 min incubation with HRP Polymer Reagent 3 (ZytoChem Plus HRP Polymer System; Zytomed Systems, Berlin, Germany) followed. For visualization, a DAB+ Substrate chromogen system (Dako, Glostrup, Denmark) was applied and the reaction was stopped with distilled water after two minutes. This was followed by a two-minute counterstain with haemalaun and bluing in tap water. The final steps were dehydration through an ascending alcohol series, treatment with Roticlear (Carlroth, Arlesheim, Switzerland), and covering with Eukitt (Merck, Darmstadt, Germany). Between all working steps, the samples were washed with PBS.

4.2.2. CCL22 Staining

The second immunohistochemical staining followed a different protocol, as it was performed in the Institute of Pathology of the University Hospital, LMU Munich. Instead of PBS, TRIS buffer (pH = 7.5) was used for rinsing. The dewaxing was followed by heat pretreatment with Target Retrieval Solution (Agilent Technologies, Santa Clara, CA, USA). After antigen retrieval, the endogenous peroxidase was blocked with 7.5% aqueous hydrogen peroxide. The 20 min incubation with blocking serum (ImmPRESS Reagent Kit Anti-Rabbit IgG; Vector, Burlingame, USA) was followed by a 60 min incubation at RT with the primary antibody (CCL22/MDC, No 500-P107, 1:200; PeproTech, Rock Hill, USA). The sections were then incubated for 30 min with anti-rabbit Ig (ImmPRESS Reagent Kit Anti-Rabbit IgG; Vector Laboratories, Burlingame, CA, USA). Visualization with DAB+ for three minutes and counterstaining with Hematoxylin Gill's Formula (Vector Laboratories, Burlingame, CA, USA) followed. Aquatex (Merck, Darmstadt, Germany) was used for covering.

4.3. Immunofluorescence Staining

To analyze the immunofluorescence staining, the Zeiss Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) was used in conjunction with the software

AxioVision 4.8.1. Each fluorescence staining was performed on a representative portion of 10% of the respective group.

4.3.1. CCL22-CK7 Staining

The immunofluorescence staining procedure was performed in a manner similar to the aforementioned immunohistochemical staining protocol. Deparaffinization in Roticlear for 20 min was followed by rehydration through a descending alcohol series ending in distilled water. Afterwards, antigen retrieval was performed by heat pretreatment in a pressure cooker with Na-citrate-buffer (pH = 6.0) for five minutes. Ultra-Vision block (Thermofisher, Waltham, MA, USA) was applied for 15 min at room temperature. Later, incubation with primary antibodies against CCL22 and CK7 (mouse IgG1 monoclonal, OV-TL 12/30, 1:30; Novocastra Leica Biosystems, Wetzlar, Germany) for 16 h at 4 °C followed. In the next step, the slices were incubated with secondary antibodies for 30 min at RT. The secondary antibodies Cy3-labeled-Goat-anti-rabbit-IgG (1:500; Dianova, Hamburg, Germany) and Alexa-Fluor-488-labeled-Goat-anti-mouse-IgG (1:100; Dianova, Hamburg, Germany) were mixed before application. After drying in the dark, the sections were mounted with mounting medium for fluorescence containing DAPI.

4.3.2. FoxP3-TUNEL Staining

To perform the double staining of Anti-FoxP3- (mouse IgG1, monoclonal 236A/E7, 1:50; Thermofisher, Waltham, MA, USA) and TUNEL staining, another protocol was necessary. The procedure resembles immunohistochemical FoxP3 staining, although the endogenous peroxidase was not blocked. After heat pretreatment, unspecific binding sites and staining were blocked by incubation with Ultra-Vision-Protein-Block (Thermofisher, Waltham, MA, USA) for 15 min. The sections were then incubated with the primary antibody FoxP3 for 16 h at 4 °C. After washing with PBS, the secondary antibody goat-anti-mouse-IgG Cy3-labeled (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was applied for 30 min at room temperature. In the next step, the TUNEL staining was performed. TUNEL enzyme (Roche, Basel, Switzerland) and TUNEL label (Roche, Basel, Switzerland) were mixed in a ratio of 1:10 and 50µL were applied on each slide. Covered with a cover glass, the sections were incubated for one hour at 37 °C. After incubation and washing in PBS, the sections were air-dried and covered with mounting medium for fluorescence with DAPI (Vector, Burlingame, CA, USA).

4.4. Evaluation of Stainings

Different methods were used to evaluate the staining, depending on the used antibody. The CCL22 staining was primarily evaluated by two independent evaluators, using the semi-quantitative International Remmle Score (IRS). The IRS was calculated by multiplying the percentage of positively stained cells (0 = no staining, 1 ≤ 10%, 2 = 11–50%, 3 = 51–80%, 4 > 80% of cells stained) in the examined tissue type, and the staining intensity (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). The IRS was determined separately for the syncytium and the decidua, with the entire slide being evaluated. In addition, a software-related evaluation was performed using the open-source software QuPath (version 0.3.0; Github, San Francisco, CA, USA). For this purpose, three images were taken of both the syncytium and the decidua of the slide under investigation at a 6.3× magnification (Flexcam C1, Leica microsystems, Wetzlar, Germany). Subsequently, the sole DAB staining was isolated in each image and the mean intensity per pixel was analyzed. For the overall analysis, the mean value for each of the three images per tissue type was calculated.

For the analysis of Tregs, the FoxP3-positive cells were counted in three randomly selected visual fields of the decidua at 25× magnification in the immunohistochemical

staining and at 20× magnification in the immunofluorescence staining and the average was calculated.

4.5. Cell Culture of Tregs and Gal-2

Tregs were isolated from PBMC (peripheral blood mononuclear cells) of human donor blood with the MACS CD4+ CD25+ CD127dim/− human regulatory T cell isolation kit II human (Nr. 130-094-775, Miltenyi Biotec, Bergisch Gladbach, Deutschland). The purity of Tregs was verified by flow cytometry analysis using the BD LSRFortessa Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) (Figure S14). The antibodies that were used for the FACS verification are listed in Table S2. The cell culture was performed as a biological triplicate, meaning that the Tregs were isolated from buffy coats of three different healthy donors. Basically, 200,000 freshly isolated Tregs were seeded in one ml RPMI-1640-medium per well of a 24-well plate. The effect of Gal-2 on the isolated and FasL-pretreated Tregs was analyzed. Two groups of Tregs were seeded for this purpose, in addition to the control group with untreated Tregs. In the first group, apoptosis was induced by using one µg/mL FasL (Treg + FasL); in addition, one µg/mL Gal-2 was added to one µg/mL FasL (Treg + FasL + Gal-2) for the second group. After five hours of incubation, the cells were extracted for the subsequent caspase-3 ELISA.

4.6. Caspase 3 ELISA

To analyze the amount of apoptotic Tregs after cell culture with Gal-2, the amount of active Caspase 3 was measured after cell extraction with the human active Caspase-3 immunoassay Quantikine ELISA (R&D Systems, Minneapolis, MN, USA). For a more accurate result, each sample was analyzed in technical triplicates.

4.7. Statistical Analysis

Statistical analysis was performed using the PC software SPSS (version 24; IBM, Armonk, NY, USA). Non-parametric tests, such as the Mann-Whitney U-test and the Spearman-Rho correlation test, were used, as the values could not be assumed to have a normal distribution. The results are given as mean value ± standard deviation. The correlation coefficient r indicates the strength of the correlation ($r < 0.3$ weak relation, $r > 0.3$ medium relation, $r > 0.5$ strong relation) [97]. In order to analyze a possible effect of the weeks of gestation, a linear regression was performed. The significance level for all tests was assumed at $p < 0.05$. In addition, an analysis of matched data regarding the weeks of gestation was conducted, using the Wilcoxon rank test, as shown in the Supplementary Data.

5. Conclusions

In summary, our results show that Tregs undergo apoptosis during PE, which may be prevented by Gal-2. Furthermore, we detected an increased expression of CCL22 in PE placentas, while Treg infiltration was reduced, indicating a positive feedback loop. Whether Gal-2 might be a potential therapeutic target to avoid Treg apoptosis, and therefore prevent an immunomodulatory imbalance during PE, needs to be further investigated in additional research.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/article/10.3390/ijms23031880/s1

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available to ensure privacy of the participants.

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Abbreviations

CCL22	CC-chemokine-ligand 22
CCL3	CC-chemokine-ligand 3 also known as macrophage inflammatory protein 1-alpha
CCL4	CC-chemokine-ligand 4
CCL5	CC-chemokine-ligand 5
CCR4	CC-chemokine-receptor 4
CX3CL1	C-X3-C-chemokine-ligand 1 also known as Fractalkine
CK7	cytokeratin 7
DAB	chromogenic 3,3′-diaminoenzidine
DAPI	4′,6-Diamino-2-phenylindile
ELISA	Enzyme-Linked Immunosorbent Assay
EVT	extravillous trophoblast
FasL	FAS ligand
FACS	fluorescence-activated cell sorting
FoxP3	forkhead box protein 3
Gal-2	galectin 2
hCG	human chorionic gonadotropin
IRS	immunoreactive Score
MACS	magnetic cell separation
MDC	macrophage derived chemokine, CCL22
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	preeclampsia (respectively: preeclampsia placentas)
Treg	regulatory T cells, respectively the group of untreated regulatory T cells
TUNEL	TdT-mediated dUTP-biotin nick end labeling

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VII. Paper IV

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Article

Gal-2 Increases H3K4me³ and H3K9ac in Trophoblasts and Preeclampsia

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Abstract: Preeclampsia (PE) is a severe pregnancy disorder with a pathophysiology not yet completely understood and without curative therapy. The histone modifications H3K4me³ and H3K9ac, as well as galectin-2 (Gal-2), are known to be decreased in PE. To gain a better understanding of the development of PE, the influence of Gal-2 on histone modification in trophoblasts and in syncytialisation was investigated. Immunohistochemical stains of 13 PE and 13 control placentas were correlated, followed by cell culture experiments. An analysis of H3K4me³ and H3K9ac was conducted, as well as cell fusion staining with E-cadherin and β-catenin—both after incubation with Gal-2. The expression of H3K4me³ and H3K9ac correlated significantly with the expression of Gal-2. Furthermore, we detected an increase in H3K4me³ and H3K9ac after the addition of Gal-2 to BeWo/HVT cells. Moreover, there was increased fusion of HVT cells after incubation with Gal-2. Gal-2 is associated with the histone modifications H3K4me³ and H3K9ac in trophoblasts. Furthermore, syncytialisation increased after incubation with Gal-2. Therefore, we postulate that Gal-2 stimulates syncytialisation, possibly mediated by H3K4me³ and H3K9ac. Since Gal-2, as well as H3K4me³ and H3K9ac, are decreased in PE, the induction of Gal-2 might be a promising therapeutic target.

Keywords: preeclampsia; histone modification; H3K4me³; H3K9ac; galectin-2; syncytialisation; trophoblasts

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1. Introduction

With an incidence of 2–5% in Europe and the USA, preeclampsia is one of the most common pregnancy disorders [1,2]. This disease is responsible for 10–15% of all maternal deaths worldwide, making it a leading cause of maternal, perinatal and neonatal morbidity and mortality [3,4].

Preeclampsia is diagnosed when arterial hypertension exceeds 140/90 mmHg in association with at least one additional organ manifestation after the 20th week of pregnancy—for example, damage to the kidneys (proteinuria > 300 mg/24 h), the liver, the lungs or the central nervous system [1,2]. The time criterion of a manifestation after the 20th week of pregnancy was selected since a previous occurrence is indicative of pre-existing organ damage [1].

The specific pathophysiological cause of preeclampsia has not been fully clarified yet. However, increased apoptosis of extravillous trophoblasts (EVT) [5,6] and a dysfunctional

placental immune response [7,8] appear to result in impaired trophoblast invasion into uterine tissue [9]. Impaired transformation of the uterine spiral arteries has also been shown to play a major role in the development of preeclampsia, being also associated with impaired trophoblast invasion and inflammatory immune regulations [2,10–12]. As a consequence, uteroplacental malperfusion occurs [9,13,14], which can lead to placental insufficiency and chronic ischemia [15], resulting in oxidative stress, hypoxic conditions and the release of anti-angiogenic factors, possibly associated with an impaired syncytialization, leading to local and systemic inflammatory reactions [10,11,16,17], all of which result in such typical symptoms as increased blood pressure and organ manifestations, e.g., proteinuria.

While there is currently no known causal therapeutic strategy for the treatment of preeclampsia [13], approaches with acetylsalicylic acid and antihypertensives exist [1,18,19]. Still, the only actual curative treatment for preeclampsia is the termination of the pregnancy and the removal of the placenta [3,18,20]. The lack of a specific therapy increases the importance of research to determine the cause of preeclampsia and discover targets for future therapy.

Long-term effects of preeclampsia for mother and fetus provide evidence of epigenetic changes in preeclampsia, including an increased risk of cardiovascular and metabolic disease, such as chronic ischemic heart disease, hypertension and type 2 diabetes [11,18,21–23]. In accordance with our previously published results showing that the transcription-promoting histone modifications H3K4me^3 and H3K9ac are decreased in trophoblasts in preeclampsia [24], several groups have already shown epigenetic alterations during preeclampsia [11,16,22,25–27]. However, since the mere identification of the involved histone modifications does not offer insight into potential treatments, the investigation of influencing factors on these processes seems to be of more relevance to evaluate possible therapeutic options.

Galectins are part of the lectin family and bind to β-galactoside units [28–32]. They are generally upregulated in various pathological situations, such as inflammation and infection [31]. Furthermore, they show a wide intra- and extracellular expression [33], for example, in the placenta [28,29]. Galectin-2 (Gal-2) is known to be expressed by both human trophoblasts and trophoblast-cell-lines, such as BeWo cells [28].

The investigated Gal-2 is considered to be a stimulator of vascular growth and a regulator of metabolism [30,34]. It shows immunomodulatory effects by inducing apoptosis in T-cells and promoting macrophage differentiation [30,31,34–36]. Gal-2 also decreases the release of proinflammatory cytokines [36] and induces trophoblast invasion [30]. Several studies have already shown a reduced expression of Gal-2 in preeclampsia, both in peripheral maternal blood and in the placenta [30,34,37,38].

Up to now, there are no data available concerning the connection of histone modifications with Gal-2. A clear connection, however, was shown by the investigation of the expression of galectin-1 (Gal-1) after treatment of neoplastic cells with histone deacetylase (HDAC) inhibitors. HDAC inhibitors induce the expression of Gal-1 [39–42] and an overexpression of HDAC1 was shown to suppress expression of LGALS1, the gene coding for Gal-1 [43], while histone deacetylation leads to downregulation of the receptor of Gal-1 [44]. Furthermore, hyperacetylation of histones is known to lead to active transcription [15,16,25,26,45]. While histone modifications do not only affect the expression of Gal-1, there is also evidence for Gal-1 influencing histone modifications. Thus, Fan et al. [46] investigated the role of Gal-1 in hypertrophic cardiomyopathy. Their cell culture experiments demonstrated that overexpression of Gal-1 attenuated isoproterenol-induced hypertrophy of myocytes via inhibition of translation and reduction of phosphorylation of the histone deacetylase HDAC4. In addition, Maier et al. [42] demonstrated that histone deacetylase inhibition could only be induced in glioma cells showing strong Gal-1 activity, not in H4IIe-hepatoma-cells, which do not overexpress Gal-1.

Gal-1 is functionally similar to Gal-2. Both proteins are prototypical galectins that are symmetric homodimers [32,47,48]. Furthermore, they share 43% of their amino acid sequences, have similar carbohydrate binding sites and show partially similar staining profiles and similar immunological and metabolic effects [31,47–49].

Due to the similarities of Gal-1 and Gal-2 in their amino acid sequences and functions, an analogy may also be expected in the case of histone modifications. Therefore, the examination of a connection between Gal-2 and the histone modifications H3K4me^3 and H3K9ac in preeclampsia could prove to be illuminating.

Different studies have shown reduced expression of Gal-2 in the placenta and an impaired syncytialisation during preeclampsia. As a result, we hypothesized that Gal-2 might influence syncytialisation through the regulation of H3K4me^3 and H3K9ac.

Therefore, the aim of this study was to analyze the effect of Gal-2 on the histone modifications H3K4me^3 and H3K9ac in BeWo and HVT cells as well as its influence on syncytialisation.

2. Materials and Methods

2.1. Sample for Statistical Analyses

To investigate possible relationships between the histone modifications H3K4me^3 and H3K9ac and Gal-2, data on immunohistochemical staining of placentas impaired by preeclampsia from previously published research projects [24,30] were used. Each staining was scored separately with the immunoreactive score (IRS) in the decidua and the syncytium. Due to former research projects, the size of the investigated groups was predetermined, which is why we analyzed the IRS of 13 placentas of women with preeclampsia and 13 control placentas for statistical correlations. Further information as for example the weeks of gestation and maternal age of the used placentas is provide in the Supplementary Material (Table S1). The analyzed tissue was collected at the University Hospital of the Ludwig-Maximilians University of Munich between 2007 and 2012.

2.2 Cell Culture

Cell culture experiments were performed to investigate the effect of Gal-2 on histone modification. For this reason, BeWo and HVT (human villous trophoblasts; ScienCell Research Laboratories, Carlsbad, CA, USA) cells were used. All cell culture experiments were performed as technical triplicates. Cells were cultured in RPMI-1640 + 10% FCS.

BeWo cells are originally derived from gestational chorionic carcinomas and are widely established as a trophoblast model cell line. Moreover, they are histologically classified as mitotic active cytotrophoblasts with a moderate percentage of syncytial differentiation and therefore are similar to the syncytiotrophoblast [50,51]. Furthermore, BeWo cells have similar expression patterns of trophoblast-specific antigens and various receptors as human trophoblasts [50,52].

HVT cells are a trophoblast cell line derived from human villous trophoblasts [53,54] also known as human cytotrophoblasts [55,56]. Cytotrophoblasts predominantly form the basal layer of the syncytium, but also migrate early in gestation to form EVTs in the decidua [16,18].

Per chamber of a four-well chamber slide, 50,000 BeWo or 50,000 HVT cells were seeded in 1 mL of growth medium (RPMI-1640 + 10% FCS). After the cells had adhered to the slide overnight, they were incubated for 32 h with recombinant human Gal-2 (Novus Biologicals, Littleton, CO, USA). Gal-2 was added in three different concentrations: 1 µg/mL, 0.1 µg/mL and 0.01 µg/mL. After the incubation, the cells were stained on the slide.

2.3. Immunocytochemical Staining

After Gal-2 incubation, all chamber slides were washed for 5 min with PBS and put in an ice-cold mixture of 50% methanol and 50% ethanol for 10 min. Afterwards, the slides were air-dried and washed with PBS and incubation with blocking solution followed (five minutes with ZytoChem Plus HRP Polymer System, mouse/rabbit—Zytomed Systems, Berlin, Germany—for the BeWo cells; 15 min with Ultra-V-Block—Thermo Fisher Scientific, Waltham, MA, USA-for the HVT cells). The primary antibodies (H3K4me^3/H3K9ac/E-cadherin + β-Catenin; Table 1) were added and incubated for 16 h at 4 °C. Primary antibodies were diluted in PBS for the BeWo cells and in DAKO dilution medium (Agilent

Technologies, Santa Clara, CA, USA) for the HVT cells. Since the procedure varies from this step for the BeWo and HVT cells, it is described separately for each. Between the single steps, the slides were washed with PBS.

Table 1. Antibodies used in immunocytochemistry of BeWo and HVT cells.

Antibody	Species Isotyp	Company	Dilution
H3K4me^3	Rabbit IgG polyclonal	Abcam ab8580	1:100
H3K9ac	Rabbit IgG monoclonal, Y28	Abcam ab3129	1:200
Goat-Anti-Mouse Alexa Fluor 488	Goat IgG, polyclonal	Dianova 115-226-062	1:100
Goat-Anti-Rabbit Cy3	Goat IgG, polyclonal	Dianova 111-165-144	1:500
E-Cadherin	Mouse IgG1, monoclonal HECD-1	Calbiochem	1:50
β-Catenin	Rabbit IgG, polyclonal	Diagnostic BioSystems	1:50

2.3.1. Staining Protocol for the BeWo Cells

The overnight incubation of the BeWo cells with the particular primary antibody was followed by a 20 min incubation with post-block reagent (ZytoChem Plus HRP Polymer System, mouse/rabbit; Zytomed Systems, Berlin, Germany) and a 30 min incubation with HRP polymer reagent (ZytoChem Plus HRP Polymer System, mouse/rabbit; Zytomed Systems, Berlin, Germany). Afterwards, chromogenic 3,3Diaminobenzidine (DAB+; Agilent Technologies; Santa Clara, CA, USA) reagent was added for 30 s before stopping the reaction in distilled water. For counterstaining, Mayer's acidic haematoxylin was used for 90 s and cells were blued in tap water for 60 s afterwards. To cover the slides, the aqueous mounting medium Aquatex (Merck, Darmstadt, Germany) was used.

2.3.2. Staining Protocol for the HVT Cells

After pretreatment and overnight incubation with primary antibodies (simultaneous primary antibodies, as for BeWo cells) a 30 min incubation with the secondary antibodies (Cy3-labeled goat-anti-rabbit IgG and Alexa Fluor 488-labeled goat-anti-mouse IgG; Table 1) at room temperature followed. Regarding the staining of histone modifications, only Cy3-labeled goat-anti-rabbit IgG was used. Slides were washed in between for 5 min with PBS.

After the incubation with the secondary antibodies, the cells were air-dried in the dark and covered with mounting medium containing DAPI to stain the nuclei for fluorescence.

2.4. Evaluation of Staining

Regarding the staining of the histone modifications H3K4me^3 and H3K9ac in BeWo and HVT cells, a scoring system similar to the semi-quantitative immunoreactive score—which is widely used in immunohistochemical analysis—was used. This modified IRS was established by Kakkassery et al. [57]. Each slide was divided into four sectors which were scored separately from 0 to 3 (0 = no signal/not a single cell was stained; 1 = minor signal/cells were stained sporadically; 2 = moderate to high signal/a couple of cells were stained within a cell layer; 3 = high signal/a couple of cells were stained within different cell layers). The cell fusion staining was evaluated by intensity measurement using the software Zeiss ZEN (blue edition; Carl Zeiss AG, Oberkochen, Germany), since this staining is not restricted to the nucleus. For this purpose, both the raw data and the data averaged for the respective DAPI intensities were analyzed.

2.5. Statistical Analysis

For the statistical analysis, the software SPSS (version 26; IBM, Armonk, NY, USA) was used. Due to the lack of normally distributed variables, the non-parametric Mann–Whitney U testand the Kruskal–Wallis test—for differences in the mean values—and the Spearman's Rho—for correlation analyses of H3K4me^3 and H3K9ac and Gal-2—were used. Significance was assumed at $p < 0.05$. The correlation coefficient r was interpreted as follows: $r < 0.3$ weak correlation, $r < 0.5$ moderate correlation, $r > 0.5$ strong correlation. All values are given as means ± standard deviations.

3. Results

3.1. The Histone Modifications H3K4me^3 and H3K9ac Correlate Significantly with Gal-2

Since both analyzed histone modifications (H3K4me^3 and H3K9ac) and Gal-2 are significantly decreased in preeclampsia [24,30], the proteins were first examined for possible statistical correlations of their immunohistochemical staining results (Table 2).

Table 2. Correlations of the histones with other proteins. The upper value is the correlation coefficient r; the second value is the *p*-value.

		Gal-2 in All Placentas		Gal-2 Only in Controls		Gal-2 Only in PE	
		Syn	Dec	Syn	Dec	Syn	Dec
H3K4me^3	Syn	0.486 *	0.450 *	0.390	0.366	0.247	0.285
		0.012	0.021	0.188	0.219	0.416	0.345
	Dec	0.402 *	0.286	0.193	0.234	0.322	0.126
		0.042	0.156	0.527	0.441	0.283	0.682
H3K9ac	Syn	0.463 *	0.368	0.019	0.020	0.556 *	0.456
		0.017	0.065	0.951	0.948	0.048	0.117
	Dec	0.559 **	0.545 **	0.197	0.151	0.759 **	0.752 **
		0.003	0.004	0.519	0.621	0.003	0.003

Correlation analysis was performed with the Spearman's Rho: * significant ($p < 0.05$); ** highly significant ($p < 0.01$). Dec, decidua; Syn, syncytium.

Considering all samples in total, significant correlations between Gal-2 in the syncytium and H3K4me^3 and H3K9ac are shown in both the syncytium ($r_{H3K4me3} = 0.486$, $p_{H3K4me3} = 0.012$; $r_{H3K9ac} = 0.463$, $p_{H3K9ac} = 0.017$) and the decidua ($r_{H3K4me3} = 0.402$, $p_{H3K4me3} = 0.042$; $r = 0.559$, $p_{H3K9ac} = 0.003$). In addition, decidual Gal-2 expression correlates with the methylated histone H3K4me^3 in the syncytium ($r = 0.450$, $p = 0.021$) and the acetylated histone H3K9ac in the decidua ($r = 0.545$, $p = 0.004$).

When controls and placentas impaired by preeclampsia were examined separately, altered results were detected. While there were no significant correlations found between the histones and Gal-2 among the controls (Table 2), Gal-2 correlated significantly with the histone H3K9ac in placentas impaired by preeclampsia. Significant correlations were found between syncytial Gal-2 and H3K9ac in the syncytium ($r = 0.556$, $p = 0.048$) and decidua ($r = 0.759$, $p = 0.003$). In addition, Gal-2 correlated significantly with H3K9ac when the decidual expressions were considered ($r = 0.752$, $p = 0.003$).

3.2. Gal-2 Promotes the Histone Modifications H3K4me^3 and H3K9ac in BeWo Cells

The immunocytochemical staining of BeWo cells showed an increased staining intensity and a higher number of stained cells after incubation with Gal-2 (Figure 1).

The examination of the trimethylated histone H3K4me^3 showed an increase in histone modification after incubation with Gal-2 ($p = 0.001$, Mann–Whitney U test). Moreover, the differences in H3K4me^3 expression were found to be significant regardless of the added concentration of Gal-2(p=0.003, Kruskal-Wallis test). Furthermore, a significant correlation between the level of H3K4me^3 and the amount of Gal-2 was found ($r = 0.962$, $p < 0.001$, Spearman's Rho).

The investigation of the acetylated histone showed similar results. Both the comparison of the control versus incubation with Gal-2 (p = 0.004, Mann–Whitney U test), as well as the comparison of all groups with different amounts of Gal-2 (p = 0.003, Kruskal–Wallis test) showed significant differences. Additionally, the expression of H3K9ac was significantly positively correlated with the amount of Gal-2 used for incubation (r = 0.956, p < 0.001, Spearman's Rho).

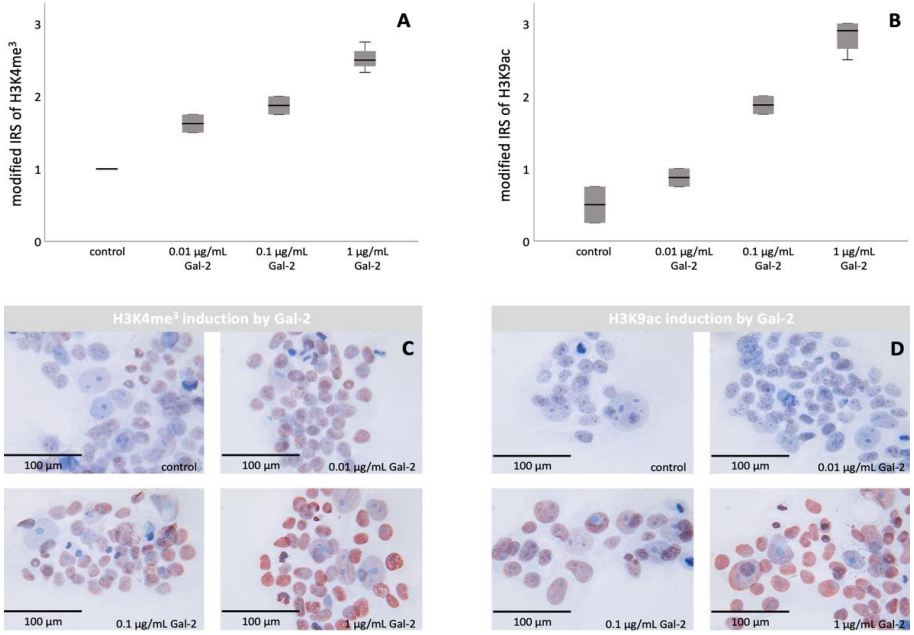

Figure 1. Induction of H3K4me^3 and H3K9ac by increasing the concentration of Gal-2 in BeWo cells: staining results of immunocytochemistry of BeWo cells. (**A**,**B**) Representation of the staining data as boxplots. (**C**) Representative images of the staining of H3K4me^3 after incubation with increasing Gal-2 concentrations. (**D**) Representative images of the staining of H3K9ac after incubation with increasing Gal-2 concentrations. Scale bar: 200 µm.

3.3. Gal-2 Promotes the Histone Modifications H3K4me^3 and H3K9ac in HVT Cells

Based on the results of the BeWo cell culture, incubation with Gal-2 was repeated with HVT cells (Figure 2).

Regarding H3K4me^3, a significant difference was found after incubation with Gal-2 compared to the untreated control group (p = 0.020, Mann–Whitney U test). Furthermore, there was a significant difference regarding all individual groups (p = 0.044, Kruskal–Wallis test) and a significant correlation between the amount of Gal-2 and histone modification (r = 0.735, p < 0.001, Spearman's Rho).

The comparison of HVT cells incubated with and without Gal-2 revealed a non-significant difference regarding the expression of H3K9ac (p = 0.684, Mann–Whitney U test). In addition, no significant difference could be found between the individual groups (p = 0.900, Kruskal–Wallis test), nor was a correlation detected between Gal-2 and H3K9ac (p = 0.769, Spearman's Rho). Since no significant results regarding H3K9ac could be found, a further descriptive analysis was conducted. H3K9ac showed increased expression after treatment with Gal-2 (1.52 ± 0.822) compared with the respective untreated control cells (1.25 ± 0.540). Furthermore, a slight elevation of H3K9ac in connection with the amount of

added Gal-2 (0.01 µg/mL = 1.50 ± 1.021; 0.1 µg/mL = 1.50 ± 0.540; 1 µg/mL = 1.56 ± 1.068) was shown.

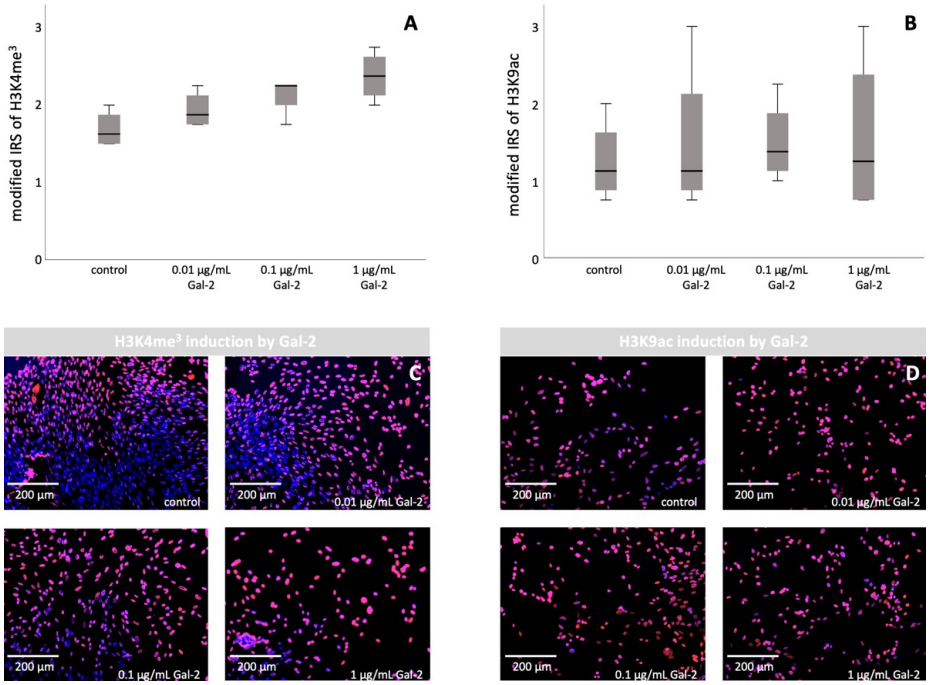

Figure 2. Induction of H3K4me³ and H3K9ac by increasing the concentration of Gal-2 in HVT cells: staining results of immunocytochemistry of HVT cells. (**A**,**B**) Representation of the staining data as boxplots. (**C**) Representative images of the staining of H3K4me³ after incubation with increasing Gal-2 concentrations. (**D**) Representative images of the staining of H3K9ac after incubation with increasing Gal-2 concentrations. Scale bar: 200 µm.

3.4. Gal-2 Promotes Cell Fusion in HVT Cell Culture

Cell fusion staining of HVT cells was performed by the visualization of E-cadherin and β-catenin after incubation with Gal-2 (Figure 3). In this staining, cell fusion is characterized by reduced expression of E-cadherin and β-catenin [29]. Certain structural proteins, such as E-cadherin, are known to be decreased after cell fusion [58–60].

The staining of E-cadherin and β-catenin showed a significantly reduced intensity after incubation with Gal-2, both in the mean intensity (E-cadherin: control = 3226.19 ± 210.494, with Gal-2 = 2714.84 ± 228.327, p = 0.018; β-catenin: control = 2396.36 ± 370.594, with Gal-2 = 1897.21 ± 392.687, p = 0.145; Mann–Whitney U test) and in the mean intensity related to DAPI (E-cadherin: control = 8.79 ± 1.924, with Gal-2 = 5.86 ± 0.578, p = 0.009; β-catenin: control = 6.39 ± 0.389, with Gal-2 = 4.05 ± 0.717, p = 0.009; Mann–Whitney U test).

Furthermore, an analysis of controls versus the individual concentrations of Gal-2 showed significant results as well. Even though the results for β-catenin were only significant when related to DAPI (p = 0.043, Kruskal–Wallis test), E-cadherin showed a significant difference in overall mean intensity (p = 0.030, Kruskal–Wallis test).

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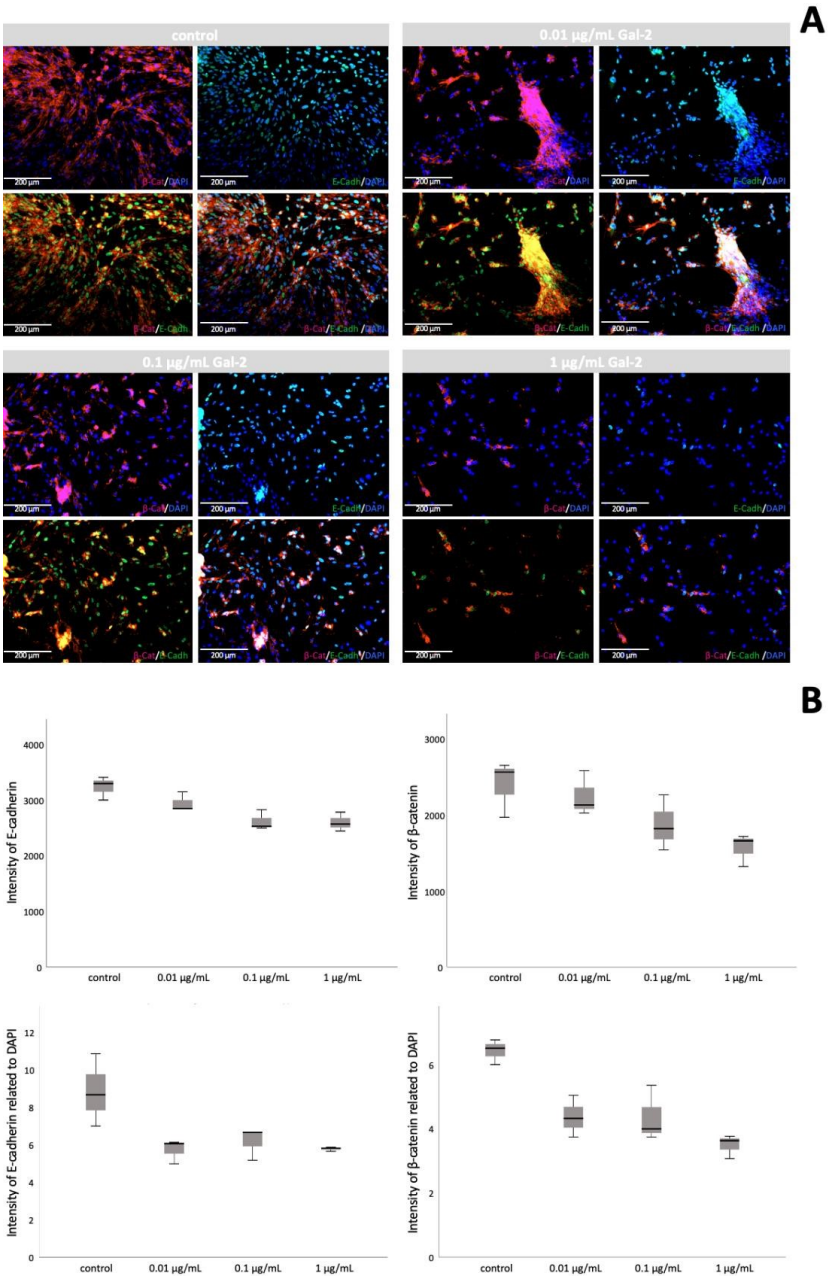

Figure 3. Induction of cell fusion by increasing the concentration of Gal-2 in HVT cells. (**A**) Exemplary images of cell fusion staining of HVT cells using β-catenin (red) and E-cadherin (green) after incubation with different concentrations of Gal-2. Nuclear staining with DAPI is shown in blue. Co-expression of β-catenin and E-cadherin results in yellowish staining. (**B**) Boxplots of the staining results of E-cadherin and β-catenin after incubation with increasing Gal-2 concentrations: mean intensity (upper row); mean intensity related to DAPI (bottom row).

4. Discussion

Preeclampsia is a serious pregnancy disorder associated with some of the highest rates of perinatal morbidity and mortality [3,4,61]. However, knowledge of the exact pathophysiology is still incomplete and, at present, only symptom-oriented therapy strategies exist [1,3,13,18–20,62]. In order to find a causal therapy for preeclampsia it is essential to determine the differences between healthy placentas and placentas affected by preeclampsia, at protein, cellular, genetic and epigenetic levels, and to investigate influencing factors.

In our present study we found a significant correlation between the histone modifications H3K4me^3 and H3K9ac and Gal-2 in placentas impaired by preeclampsia. Due to the overall positive correlations of the histone modifications and Gal-2, the influence of Gal-2 on these histone modifications was investigated in two different trophoblast cell culture models. A significant increase in the histone modification H3K4me^3 in BeWo and HVT cells cultured with Gal-2 and an increase depending on Gal-2 concentration were found. In contrast, H3K9ac was only significantly affected by Gal-2 in the BeWo cell culture, while merely a slight descriptive difference appeared in HVT cells between the control group and the groups incubated with Gal-2.

These differences depending on trophoblast cell type are of sufficient interest to be considered more in detail. While the underlying pathophysiological mechanism is not yet known, different characteristics of the cell lines allow possible conclusions to be drawn. Whilst HVT cells resemble cytotrophoblasts [55,56,63], BeWo cells have several characteristics of cytotrophoblasts but also functional similarities with the syncytiotrophoblast [51]; to a certain extent, they also exhibit fusiogenic properties [50,64].

To interpret our results properly, one has to consider the structure of the placenta and the different functions of various differentiated trophoblast types in the organ. The placenta connects fetal tissue to the uterine wall and exhibits tumor-like properties [65]. It develops from the fifth day after fertilization with the differentiation of trophoblasts, which are epithelial cells, with partially highly proliferative and invasive growth [18,66,67]. Starting at the time of implantation, the trophoblasts proliferate and fuse, resulting in the syncytiotrophoblast, which has no lateral cell borders, thus representing a single multinucleated cell [16,18,68]. During pregnancy, villous cytotrophoblasts continuously fuse with the syncytiotrophoblast and lose their proliferative activity [18]. The syncytiotrophoblast forms a boundary layer around the fetal tissue and controls maternofetal transfer [2,16,18]. Another part of the cytotrophoblast migrates into the decidua, leading to the formation of EVTs, which arrange themselves into cell columns and subsequently also lose their ability to proliferate [18].

Our immunohistochemical placenta staining showed a more prominent correlation of histone modifications and Gal-2 in the syncytium, which is consistent with the results of the cell culture experiments, showing an effect of Gal-2 on histone modifications in BeWo cells, exhibiting functional similarities with the syncytiotrophoblast. These results point to a significant role of Gal-2 possibly related to the analyzed histone modifications in the syncytium during preeclampsia. These findings are consistent with data found in the literature indicating diminished syncytialisation in preeclampsia [69]. Various acetylated histones, e.g., H3K9, H3K14 and H3K27, are known to be involved in impaired angiogenesis and syncytialisation in placental ischemia or hypoxia [25]. Moreover, H3K4me^3 and H3K9ac are known to be related to syncytialisation. Since both histone modifications indicate active regions at gene enhancers and promoters [70,71], it could be hypothesized that they may be upregulated after successful syncytialisation, as was already demonstrated in BeWo cells [60].

Syncytiotrophoblast fusion occurs throughout pregnancy [18]. Gal-1, which is closely related to Gal-2 [31,48], leads to the stimulation of syncytium formation [29,38,72]. Therefore, to evaluate the influence of Gal-2 on histone modification more accurately, cell fusion and thus syncytialisation were examined in the present study, revealing increased cell fusion depending on the amount of Gal-2 added in culture.

Gal-2 is already known to be involved in placental formation and is expressed by cytotrophoblasts in the placenta of healthy women [37]. Furthermore, Gal-2 has an additional effect on placental angiogenesis [30,34] and it is known to be strongly reduced in the placentas of patients impaired by preeclampsia, as well as in the peripheral blood of women suffering from preeclampsia [30,37]. To investigate syncytialisation, we chose HVT cells, since BeWo cells themselves show fusiogenic potential and already resemble the syncytiotrophoblast. However, since we wanted to study the process of syncytialisation, which basically means the fusion of cytotrophoblasts to form the syncytiotrophoblast, we chose to study HVT cells which are cytotrophoblasts.

Since both H3K4me^3 and H3K9ac are known to be activators of transcription [25,45] and have already been shown to be upregulated during syncytialisation [60,73], the results of the present study point towards an association of the histone modifications H3K4me^3 and H3K9ac with increased cell fusion via promotion of transcription. According to our in vitro experiments, the examined histone modifications are affected by Gal-2. The decreased levels of Gal-2 in preeclampsia [30] could be partially responsible for the impaired histone modifications H3K4me^3 and H3K9ac in preeclampsia [24]. Reduction of these histone modifications and Gal-2 might therefore result in a decreased syncytiotrophoblast fusion, leading to disturbed placentation [74]. Since our results showed a positive association between and a significant influence of Gal-2 on syncytialisation, our data suggest that this effect of the histone modifications may occur in interaction with Gal-2. These findings also reinforce the association between the expression of Gal-2 and preeclampsia which was postulated in previous studies [30].

Interestingly, our results indicate that H3K9ac may have a stronger influence on syncytialisation than H3K4me^3, based on the theory that Gal-2 influences syncytialisation via histone modification. On the one hand, a stronger correlation is shown between H3K9ac and Gal-2 in the immunohistochemical staining, especially when only placentas impaired by preeclampsia are considered, whereas only a moderate correlation is shown between H3K4me^3 and Gal-2. On the other hand, in the cell culture of the BeWo cells, a significant correlation with the added amount of Gal-2 was shown only with respect to H3K9ac.

Moreover, these results are in agreement with the findings of Li et al. [73], who demonstrated a specific influence of H3K9ac on syncytialisation. Assuming that H3K9ac indeed makes a greater contribution to syncytialisation, further research on whether syncytialisation can be influenced by histone deacetylases or inhibition might be worth considering.

Still, our work is not free of limitations. Even though both BeWo cells and HVT cells are recognized cell line models for the placenta, a culture of freshly isolated trophoblasts might be interesting, whereby a picture of the actual influence of Gal-2 on histone modification and syncytialisation could only be revealed in an animal model, since the further hormonal influences of the placenta are missing in a single trophoblast culture model. In addition, the different staining methods for the BeWo and HVT cells allow only limited comparability of the cell lines. Nevertheless, this choice of different staining methods was reasonable, even though immunohistochemical cell staining is more precise and less error-prone than immunofluorescence. By contrast, in the case of HVT cells we wanted to establish clear comparability with E-cadherin/β-Catenin double-staining in order to be able to draw conclusions about the influence of Gal-2 on syncytialisation via histone modification. Lastly, it remains to be noted that we have only demonstrated the respective correlations of Gal-2 with the histone modifications H3K9ac and H3K4me^3 based on immunostaining at the protein level. Moreover, in our study we did not perform a sequencing analysis to verify the effects we showed at the gene level.

Regardless of the significance of the association between Gal-2 and the examined histone modifications, our results show an influence of Gal-2 on the histone modifications H3K4me^3 and H3K9ac in trophoblasts, whereas the effect seems to be more dominant in the syncytiotrophoblast. Since Gal-2, as well as the histone modifications H3K4me^3 and H3K9ac, are decreased in preeclampsia, a possible pathophysiological involvement in the development of preeclampsia can be assumed. While further in vivo research is

needed to prove the association between Gal-2 and the histone modifications H3K4me^3 and H3K9ac found in the present study, Gal-2 seems to be a promising therapeutic target in the treatment of preeclampsia.

5. Conclusions

In this study we were able to show a connection between Gal-2 and the histone modifications H3K4me^3 and H3K9ac in trophoblast cells and preeclampsia. We postulate that Gal-2 stimulates syncytialisation, which might be mediated by H3K4me^3 and H3K9ac. Since Gal-2, as well as H3K4me^3 and H3K9ac, is decreased in PE, the induction of Gal-2 might be a promising therapeutic target in the treatment of preeclampsia.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom12050707/s1, Table S1: Gestational age and sex of the babies of healthy and PE affected pregnancies.

Author Contributions: Conceptualization, S.M. (Sarah Meister), T.K. and U.J.; methodology, L.H. and S.M. (Meister Sarah); software, L.H.; validation, M.M., A.B. and U.J.; formal analysis, L.H. and T.K.; investigation, L.H.; resources, S.M. (Sarah Meister); data curation, S.M. (Sarah Meister); writing—original draft preparation, L.H., A.B. and S.M. (Sarah Meister); writing—review and editing, L.H., S.M. (Sarah Meister), A.B., S.B. and T.K.; visualization, L.H.; supervision, S.M. (Sarah Meister), T.K. and U.J.; project administration, S.M. (Sven Mahner), A.B., S.C. and U.H.; funding acquisition, T.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the LMU Munich (reference #337-06; amended 26 August 2013).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BeWo cells	Trophoblast cell line, originates from human chorioncarcinoma
DAPI	4^0,6-diamidino-2-phenylindole
EVT	Extravillous trophoblast
FCS	Fetal calf serum
Gal-1	Galectin-1
Gal-2	Galectin-2
H3K14	Histone H3 protein, modified at the 14th lysine residue
H3K27	Histone H3 protein, modified at the 27th lysine residue
H3K4me^3	Trimethylated 4th lysine residue of the histone H3 protein
H3K9ac	Acetylated 9th lysine residue of the histone H3 protein
HDAC	Histone deacetlyase
HVT cells	Human villous trophoblast cell line
IRS	International Remmele Score
LGALS1	Gene coding for galectin-1
PE	Preeclampsia
RPMI-1640	Roswell Park Memorial Institute 1640 cell culture medium

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