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Histologische und experimentelle Untersuchung inflammatorischer und epigenetischer Dysregulation in der Plazenta bei Gestationsdiabetes

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Hiermit erkläre ich, dass die elektronische Version der eingereichten Dissertation mit dem Titel
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INHALTSVERZEICHNIS

1. EINLEITUNG

1.1. DEFINITION & EPIDEMIOLOGIE DES GESTATIONSDIABETES

Jede physiologische Schwangerschaft ist durch eine Vielzahl metabolischer Umstellungen geprägt, welche das optimale Wachstum des Feten gewährleisten sollen. Es liegt auf der Hand, dass das Risiko metabolischer Dysbalancen und Fehlregulation in diesem Rahmen hoch ist. Der Gestationsdiabetes ist unter den schwangerschafts-assoziierten Stoffwechselstörungen die häufigste weltweit [1, 2]. Gemäß aktueller S3 Leitlinie der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe (DGGG) und der Deutschen Diabetes Gesellschaft (DDG) ist Gestationsdiabetes mellitus (GDM) definiert als: „eine Glukosetoleranzstörung, die erstmals in der Schwangerschaft mit einem 75-g-oralen Glukosetoleranztest (oGTT) unter standardisierten Bedingungen und qualitätsgesicherter Glukosemessung aus venösem Plasma diagnostiziert wird." (Seite 7 [2])

Aufgrund international uneinheitlicher Diagnosekriterien, Screeningverfahren bzw. deren Fehlen, sowie demographischer Faktoren variieren die Angaben bzgl. der Inzidenz des GDM [2]. Der aktuelle Diabetes Atlas (2019) der internationalen Diabetes Föderation gibt die weltweite Prävalenz mit 12,8% (Anteil an der Gesamtzahl der Lebendgeburten) an [1]. Es bestehen jedoch erhebliche regionale Unterschiede sowohl bzgl. der aktuellen Prävalenz als auch der Prognosen zur künftigen Entwicklung [1, 3].

Insbesondere wenn der mütterliche GDM nicht adäquat eingestellt bzw. behandelt wird, es also vermehrt zu Hyperglykämien kommt, führt die Erkrankung zu zahlreichen kurzfristigen Komplikationen [4, 5]: Für die Mutter besteht unter anderem ein erhöhtes Risiko, während der Schwangerschaft eine Präeklampsie zu entwickeln (Odds Ratio (OR) 1.81) [6]. Die Feten haben insbesondere ein deutlich erhöhtes Risiko für eine Makrosomie (OR 3.43) mit oder ohne Fehlbildungen (diabetische Fetopathie) [4, 7]. Die Makrosomie zieht wiederum weitere Risiken nach sich. Zum einen findet sich eine erhöhte Sectiorate (OR 1,46) [4]. Zum anderen besteht intrapartal ein erhöhtes Schulterdystokie- und Verletzungsrisiko [7].

Von besonderem Interesse – pathophysiologisch im Sinne der Grundlagenforschung und im Rahmen der Prävention – sind außerdem die zunehmend evidenten Langzeitfolgen. GDM-Patientinnen haben ein deutlich erhöhtes Risiko im Verlauf ihres Lebens an Diabetes Mellitus Typ 2 (DM2) zu erkranken (Relatives Risiko 7.43) [8]. Vereinzelt konnte gezeigt werden, dass dieses Risiko durch postpartale Lebensstilinterventionen gesenkt werden konnte, jedoch laut einer aktuellen Metanalyse ohne statistische Signifikanz [9]. Andere Studien konnten Stillen als einen protektiven Faktor identifizieren, was aufgrund der relativ einfachen Umsetzbarkeit großes präventives Potential bergen könnte [10, 11]. Tatsächlich

scheint GDM in der Vorgeschichte auch unabhängig von einer späteren DM2 Erkrankung ein Risikofaktor für Arteriosklerose zu sein [12]. Auch für die Kinder der GDM-Patientinnen ist das DM2-, Adipositas- und dadurch insgesamt das kardiovaskuläre Risiko lebenslang erhöht [13, 14].

Bereits im Jahr 2015 fielen in Deutschland 35 Milliarden US-Dollar (bzw. das entsprechende Euro-Äquivalent) für diabetesbezogene Gesundheitsausgaben an [15]. In Anbetracht der steigenden GDM-Inzidenz und damit verbunden Langzeitfolgen hat die weitere Erforschung der Pathophysiologie des GDM eine enorme präventive, gesundheitliche und ökonomische Relevanz.

1.2. Ätiologie & Pathophysiologie des Gestationsdiabetes

Im Verlauf einer physiologischen Schwangerschaft ändert sich die Glukosetoleranz der Mutter, um sich an die Bedürfnisse des Fetus anzupassen: In der Frühschwangerschaft steigt die Insulinsensitivität durch die hohen Konzentrationen von humanem Choriongonadotropin zunächst leicht an. Wenn die Plazenta zunehmend auch endokrinologisch funktionsfähig wird, dreht sich dieser Trend jedoch um. Es kommt zur gesteigerten Produktion von anti-insulinergen Hormonen, v.a. Progesteron und Humanes Plazentalaktogen (HPL). Dies führt folglich zu einer leichten Hyperglykämieneigung [7, 16]. Diese physiologischen Mechanismen sollen eine ausreichende fetale Glukoseversorgung garantieren. Kann diese feine endokrine Balance aufgrund verstärkter mütterlicher Insulinresistenz und/oder unzureichender Kompensation durch die mütterlichen β-Zellen jedoch nicht aufrechterhalten werden, kommt es zum manifesten GDM [2, 7, 17, 18].

Glukose wird konzentrationsabhängig und insulinunabhängig via GLUT-1 Transporter über die Plazenta zum Fetus geschleust, während Insulin die plazentare Schranke nicht überwinden kann [19, 20]. Die mütterliche Hyperglykämie führt folglich zu einer starken fetalen Hyperglykämie, die (mit einer gewissen Latenz) zur fetalen Hyperinsulinämie führt. Es kommt zur Störung der feto-maternalen Glukose-Insulin-Homöostase, welche wiederum zu den oben beschrieben Komplikationen wie Makrosomie führt [7].

Die entscheidenden Risikofaktoren für die Entwicklung eines GDM ähneln denen des DM2: Genetische Prädisposition (positive Familienanamnese für DM2 oder vorausgegangene GDM), präkonzeptionelle Adipositas (bzw. hoher mütterlicher Body Mass Index (BMI)) oder Insulinresistenz und höheres mütterliches Alter [21, 22]. Ein weiterer, erst seit Neuerem untersuchter Risikofaktor scheint ein präkonzeptioneller Vitamin-D-Mangel zu sein [23]. Manche dieser Risikofaktoren haben Implikationen für die zugrunde liegenden molekularen Mechanismen des GDM. Zum Beispiel ist Adipositas wiederum mit Insulinresistenz und einer chronischen Inflammation assoziiert [24]. Von

einem ganzheitlichen Verständnis dieser Mechanismen sind wir jedoch noch immer weit entfernt.

1.2.1. Die Rolle der Plazenta

Durch das Einwachsen der Blastozyste in das Stratum functionalis des Endometriums beginnt am 6.-7. Tag post conceptionem die Implantation und Plazentation. Die äußerste Schicht der Blastozyste – der fetale Trophoblast – bildet gemeinsam mit dem in Decidua umgewandelten Stratum functionalis der Mutter die Plazenta [25]. Sie ist also nicht nur die Schalt- und Kommunikationsfläche zwischen Mutter und Fetus, sondern eine Vereinigung von histologischem und genetischem Material beider. Somit ist sie auch die Schlüsselstelle der feto-maternalen Toleranz und rückt zunehmend in den Fokus immunologischer Forschung [26].

Basisfunktionen der Plazenta sind die adäquate Nährstoffversorgung des Kindes und der Abtransport der Abbauprodukte [25]. Im Hinblick auf den GDM ist besonders die plazentare Glukosetransportkapazität relevant. Hier scheint es große inter-individuelle Unterschiede zu geben, da trotz unzureichender Therapie fetale Komplikationen ausbleiben können, es jedoch auch bei angemessen therapierten Müttern zu fetaler Makrosomie kommen kann [7]. Ein besseres Verständnis der hier zugrundeliegenden Mechanismen könnte möglicherweise zu individuell angepassten Therapien führen, die fetalen Komplikationen effektiver entgegenwirken könnten.

Als Schaltfläche für den feto-maternalen Glukosestoffwechsel ist die Plazenta außerdem auch selbst von der Hyperglykämie betroffen. GDM Plazenten zeigen u.a. eine erhöhte Zahl unreifer Zotten, eine deutliche Fibrose sowie – trotz kompensatorischer Hypervaskularisation der Zotten (Chorangiose) – eine reduzierte Flusseffizienz [27]. Wenn diese morphologischen Veränderungen besonders ausgeprägt sind, können sie zur fetalen Minderversorgung und so zur intrauterinen Wachtumsrestriktion (IUWR) führen [7]. Einige lichtmikroskopische Untersuchungen deuten darauf hin, dass weder diätetische Maßnahmen noch eine Insulintherapie diese Veränderungen vollständig verhindern [28, 29].

Als Hauptproduzentin vieler anti-insulinerger Hormone (Progesteron, HPL) ist die Plazenta selbst eine der Stellschrauben in der Ätiopathogenese des GDM. Diese anti-insulinergen Hormone haben zusätzlich immunmodulatorische Effekte – stellen also potentiell eine direkte Verbindung zur inflammatorischen Komponente des GDM dar [30, 31].

Wie bereits angedeutet ist die Plazenta maßgeblich an der Regulation des Immunsystems beteiligt, die für die Aufrechterhaltung der Schwangerschaft notwendig ist. Durch die Freisetzung verschiedener Zytokine, interagieren sowohl Synzytiotrophoblast-, als auch

extravillöse Trophoblastzellen mit zahlreichen Immunzellen, u.a. natürliche Killerzellen und Makrophagen [26, 32]. Wie die plazentare Hormonausschüttung scheinen auch die sezernierten Immunmodulatoren Einfluss auf den Glukosestoffwechsel zu haben.

So zeigten Lowe, et al. [33] bei euglykämen Schwangeren eine signifikante Korrelation zwischen Entzündungsmediatoren und Glukosetoleranz. Während einer physiologischen Schwangerschaft steigt die Tumornekrosefaktor-α- (TNFα-) Konzentration an, wobei bekannt ist, dass die Plazenta TNFα produziert und dieses hauptsächlich in den mütterlichen Kreislauf sezerniert [34]. Auch TNFα ist mit dem Grad der mütterlichen Glukosetoleranz assoziiert [35]. Auf molekularer Ebene konnte gezeigt werden, dass das plazentare Transkriptom in GDM Patientinnen deutlich verändert ist [36]. Insbesondere die Expression inflammatorischer Mediatoren wie TNFα und Interleukinen war hoch reguliert. Laut Radaelli, et al. [36] könnte das daraus resultierende pro-inflammatorische Milieu sowohl zur oben beschriebenen plazentaren Umstrukturierung als auch zur Entstehung der Insulinresistenz beitragen. Weiteres dazu wird im folgenden Abschnitt detailliert besprochen.

Vor dem Hintergrund, dass viele Schwangerschaftskomplikationen eine lebenslang anhaltende Veränderung des kindlichen Risikoprofils nach sich ziehen, rücken epigenetische Mechanismen zunehmend in den Fokus der Reproduktionsmedizin [37, 38]. Es ist weitgehend etabliert, dass epigenetische Veränderungen der Plazenta im Verlauf einer physiologischen Schwangerschaft regelhaft auftreten [39]. Die mütterliche Ernährung beeinflusst das plazentare Epigenom [40] und auch bei GDM konnten epigenetische Veränderungen der Plazenta gezeigt werden [41, 42]. Die Relevanz epigenetischer Dysregulationen bei GDM wird ebenfalls im Folgenden eingehend diskutiert.

1.2.2. Inflammation & Immunmodulation – Die Rolle der Galektine

Die Rolle eines chronisch inflammatorischen Milieus in der Pathogenese der Insulinresistenz ist seit längerem Gegenstand der Diabetesforschung. Es konnte durch diverse Studien eine signifikante positive Korrelation zwischen verschiedenen Entzündungsmediatoren und dem Diabetesrisiko etabliert werden [43, 44], welche auch durch funktionelle Studien bzgl. ihrer Kausalitätsrelevanz untermauert wird [45].

Nicht überraschend spielt auch in der Pathogenese des Gestationsdiabetes eine chronische, subklinische Inflammation eine Rolle. Kirwan, et al. [34] konnten bereits 2002 TNFα als Prädiktor für GDM identifizieren. Im Verlauf konnten diese Ergebnisse untermauert und viele weitere Entzündungsmediatoren identifiziert werden, welche bei GDM dysreguliert sind [46]; insbesondere Interleukin (IL) -6, Leptin, Adiponectin und Gewebeinhibitor der Metalloprotease (TIMP) -1 scheinen von großer Relevanz zu sein

[47-50]. Letztere scheinen möglicherweise auch in der Entwicklung des langfristig erhöhten kardiovaskulären Risikos der GDM Patientinnen eine Rolle zu spielen [49].

Galektine sind per definitionem Proteine, die eine so genannte „cardohydrate binding domaine“ (CBD) enthalten (eine hoch konservierte Aminosäure Sequenz), mit der sie spezifisch an β-galaktosidhaltige Zuckerdomänen binden [51]. Anhand ihrer molekularen Struktur werden die Galektine in drei Gruppen eingeteilt: Prototyp Galektine enthalten nur eine CBD und formen daher typischerweise Dimere. Chimeratyp Galektine enthalten eine CBD und eine funktionelle N-terminale nicht-CB Domäne, während Tandem-Repeat Galektine zwei verschiedene CBDs innerhalb eines Moleküls beinhalten [52].

Die diversen Funktionen der Galektin-Proteine sind seit mehreren Jahren Gegenstand intensiver Forschung. Sie modulieren für die menschliche Physiologie grundlegende Prozesse wie Zell-Zell-Interaktion, Apoptose und Migration [53-55]. Je nach genauer Lokalisation (intra- oder extrazellulär, exprimierender Zelltyp) können Galektine nicht nur unterschiedliche, sondern z.T. konträre Effekte erzielen [56]. Davon ausgehend regulieren sie auch komplexere Vorgänge, von Angiogenese über metabolische Homöostase bis hin zur Regulation des Immunsystems [53, 57-59].

9 der 16 (aktuell identifizierten) humanen Galektine werden von der Plazenta exprimiert [60]. Insbesondere Galektin-1 und -3 wurden bereits eingehend auf ihre Funktion in der physiologischen Plazentation hin erforscht. Beide scheinen für eine zuverlässige Implantation von Bedeutung zu sein [61-63]. Es ist daher wenig überraschend, dass mehrere Studien eine Dysregulation von Galektinen in Präeklampsie-(PE-)Fällen zeigen konnten [57, 64]. Auch die Galektin-2 Expression war in Plazenten präeklamptischer Mütter signifikant erniedrigt [65]. Tatsächlich konnte Galektin-13 (auch bekannt als Plazenta Protein 13 (PP-13)) als ein neuer, bereits im klinischen Kontext einsetzbarer Biomarker zur Risikostratifizierung der PE identifiziert werden [66].

Allen voran die immunmodulatorischen Eigenschaften der Galektine sind im Kontext des GDM von großem Interesse und es wurden bereits einige Studien diesbezüglich durchgeführt. Blois, et al. [67] verglichen die Expression und Konzentration von Galektin-1 in GDM-Plazenten, respektive in den Seren der Patientinnen und gesunden Kontrollen. Sie konnten eine signifikante Hochregulation von Galektin-1 in GDM-Plazenten feststellen, die von einer signifikant niedrigeren peripheren Konzentration begleitet war. Nach aktuellem Forschungsstand wird Galektin-1 gemeinhin als anti-inflammatorisch wirksam gehandelt [53, 68]. In diesem Licht deuten Blois, et al. [67] die Hochregulation als einen Kompensationsmechanismus, um der chronischen Inflammation entgegenzuwirken. Galektin-13, ein weiteres vornehmlich anti-inflammatorisches Galektin, ist in GDM-Fällen

hingegen signifikant erniedrigt, könnte also seinerseits zur Genese der inflammatorischen Dysbalance beitragen [69].

Galektin-2 ist trotz seiner frühzeitigen Entdeckung deutlich weniger erforscht als andere Galektine [60]. Zwar gibt es erste Daten zu Galektin-2 Dysregulationen (im Sinne einer verringerten Expression) bei PE, IUWR und habituellen Aborten, jedoch gibt es bislang keinerlei Untersuchungen zu GDM. Aufgrund der vielfältigen immunmodulatorischen Effekte von Galektin-2 [70, 71] besteht jedoch eine klare Relevanz für die Pathophysiologie des GDM. Daher wurde in der vorliegenden Studie (Publikation 1) eine systematische Untersuchung der Galektin-2 Expression in GDM-Plazenten und Kontrollen durchgeführt.

1.2.3. Epigenetische Aspekte – Die Rolle der Histonmodifikationen

Bereits vor mehr als 30 Jahren zeigten Barker and Osmond [72] in ersten geographisch-epidemiologischen Studien einen Zusammenhang zwischen frühkindlichem Ernährungsstatus und kardiovaskulärem Risiko. Daran schlossen sich zahlreiche weitere Untersuchungen an. Sie führten schließlich zur Formulierung Bakers Theorie des „Developmental Origin of Human Disease“ [73]. Diese besagt, dass das intrauterine Milieu die Gesundheit bzw. Krankheit im weiteren Leben maßgeblich beeinflusst. Initial konnte dies insbesondere bei intrauteriner Nährstoffrestriktion, also niedrigem Geburtsgewicht in Verbindung mit hohem kardiovaskulärem Risikoprofil, gezeigt werden [74]. Wie bereits erwähnt gilt heute jedoch auch eine vergleichbare Korrelation zwischen fetaler Makrosomie, bzw. intrauteriner Überversorgung bei GDM und langfristigem kardiovaskulären Risiko als gesichert [14].

Die Epigenetik wird aktuell als ein wahrscheinliches molekulares Korrelat gemäß Bakers Theorie gehandelt [74, 75]. Nach Allis, et al. [76] ist das Epigenom definiert als die Summe an Chromatinmodifikationen, welche gemeinsam zu verschiedenen Genexpressionsmustern eines Genoms führen. Chromatin bezeichnet das Makromolekül bestehend aus der DNA-Doppelhelix und Histonprotein-Oktameren um welche diese gewickelt wird [77]. Diese „Verpackung“ dient nicht nur dem Schutz und der platzsparenden Aufbewahrung der DNA, sondern bildet auch die Angriffsfläche der Epigenetik [78]. Die drei wichtigsten Stellschrauben der Epigenetik sind: DNA-Methylierung, post-translationale Modifikationen (PTM) der Histone und nicht-codierende RNA (ncRNA) [76, 79]. DNA-Methylierung findet hauptsächlich an Cytosin-Guanin-Dinukleotid-(CpG)-Inseln statt. Dies führt in der Regel zur Stilllegung eines Gens. Die DNA-Methylierung scheint jedoch über Interaktionen mit anderen Mechanismen auch an komplexeren Regulationen beteiligt zu sein [75]. Über die Funktionen von ncRNA

ist noch wenig bekannt; möglicherweise können sie einen bestimmten Lokus für folgende Chromatinmodifikationen markieren [79, 80].

Seit der Entdeckung der ersten Histon-Acetyltransferase (HAT) 1996 konnte eine Vielzahl verschiedener post-translationaler Histonmodifikationen identifiziert werden [79]. PTMs sind dynamisch; sie werden durch „writer"- (z.B. HATs) und „eraser"- (z.B. Histone Deacetylasen HDACs) Enzyme an Histon Seitenketten modifiziert [76]. Ihre Funktionen erfüllen sie via *cis*- und *trans*- Mechanismen. Cis-Mechanismen beschreiben eine direkte Interaktion zwischen PTM und DNA Molekül, z.B. basierend auf der elektrischen Ladung und folglich intermolekularen Bindungen. Der indirekte *trans*-Mechanismus basiert auf der Rekrutierung von „reader"-Proteinen, die weitere Reaktionen nach sich ziehen. Zusätzlich kommt es zu Interaktionen zwischen verschiedenen PTMs, die sich gegenseitig verstärken oder abmildern können [76, 81, 82]. Basierend auf diesem sogenannten „cross-talk" und der daraus resultierenden Komplexität, wurde die Hypothese vorgebracht, dass die Summe aller Histonmodifikationen einen gewissen „Histon-Code" darstellt [83].

Die Rolle epigenetischer Mechanismen in der Pathophysiologie des GDM war und ist Gegenstand intensiver Forschung. Frühe Untersuchungen basierten auf Zellkultur- und Tiermodellen und waren insbesondere als Basis für die Formulierung zentraler Hypothesen entscheidend (siehe [41, 84] für Reviews). Insbesondere sei hier die These zu nennen, dass das erhöhte kardiovaskuläre Risiko der Nachkommen in Folge des ungünstigen intrauterinen Milieus durch epigenetische Prägungen vermittelt wird. Mittlerweile hat die Zahl humaner Studien, welche diese These untermauern, zugenommen. Diese wurden 2017 durch Moen, et al. [85] und 2019 nochmals durch Elliott, et al. [86] in systematischen Reviews zusammengefasst. (Hier ist auch zu bemerken, dass sich die Zahl der relevanten Studien innerhalb der zwei zwischen den Reviews liegenden Jahre mehr als verdoppelt hat). Aufgrund der Fülle an Studien, sollen im Folgenden nur die im Kontext dieser Dissertation relevantesten Ergebnisse kurz dargelegt werden. Die Mehrheit der Studien befasst sich mit Unterschieden in DNA-Methylierung, wobei deskriptive assoziationsbasierte Studien überwiegen [86]. Durch umfangreichere Studiendesigns und statistische Auswertung können z.T. kausale Rückschlüsse gezogen werden. Côté, et al. [87] untersuchten in ihrer Kohortenstudie sowohl plazentare DNA-Methylierungsmuster stoffwechselrelevanter Gene, als auch die Konzentration korrespondierender Proteine im Nabelschnurblut und zeigten mittels statistischer Mediationsanalyse eine (partielle) Vermittlung der hyperglykämischen Folgen durch DNA-Methylierung. Eine weitere Studie an neonataler DNA zeigte vergleichbare Veränderung in der DNA-Methylierung nach intrauterinem Diabetes (GDM oder vorbekanntem DM), welche wiederum als prädiktiver Marker des zukünftigen Diabetesrisikos fungieren konnten [88].

Untersuchungen zu Histonmodifikationen sind weniger zahlreich, insbesondere im Menschen. In Zellkultur- und Mausmodellen konnte gezeigt werden, dass kurzzeitige Hyperglykämiespitzen zu einer epigenetisch-vermittelten Änderung der Genexpression in Endothelzellen führten und diese über mehrere Tage anhielten [89]. Weitere Daten lieferte z.B. ein Primatenmodell [90]: Hier konnten signifikante Auswirkungen präkonzeptioneller und pränataler Ernährung – in diesem Fall hochkalorische, fettreiche Nahrung – auf die kindliche Chromatinstruktur (Histon 3 Acetylierungen) und die korrespondierenden Enzyme (HDAC 1) gezeigt werden. Insbesondere waren Genloki des Glukosestoffwechsels von diesen epigenetischen Veränderungen betroffen.
Beim Menschen konnte eine kleine Fall-Kontroll-Studie signifikante Veränderungen der Histonmethylierungen in GDM Patientinnen zeigen, die im weiteren Verlauf an DM2 erkrankten [91]. Eine weitere Studie an mütterlichem Serum und Monozyten beschrieb eine herabgesetzte HDAC 2 Aktivität in GDM Patientinnen, welche mit einer mitochondrialen Dysfunktion einherging [92].
Es gibt also einige Daten zu Histonmodifikationen in GDM auf mütterlicher Seite. Die hier vorgelegte Studie (Publikation 2) war jedoch die erste Studie, die auch humanes fetales Gewebe auf globale Dysregulationen von (aktivierenden) Histonmodifikationen untersuchte.

1.3. Zielsetzung & Ergebnisse

Sowohl in der Erforschung von Insulinresistenz generell, als auch speziell auf GDM bezogen, rücken zwei große Achsen zunehmend in den Fokus: 1) Chronische subklinische Inflammation und die damit verbundenen zellulären und humoralen immunmodulatorischen Signalwege. 2) Epigenetische Mechanismen und die damit einhergehenden Veränderungen von Genexpressionsmustern. Besonders interessant ist, dass beide Achsen für die Pathophysiologie des langfristig erhöhten Risikoprofils von Müttern und Kindern relevant zu sein scheinen [49, 87]. Außerdem wirken sich beide Achsen auf die Plazenta aus und sind somit gut an dieser zu untersuchen.
Aufgrund ihrer immunologischen Relevanz sind bereits mehrere Galektine bzw. deren Expressionsmuster in GDM Fällen untersucht worden [67, 69]. Basierend auf deskriptiven Studien, wurde Galektin-13 kürzlich als potentieller prädiktiver Biomarker identifiziert [93]. Trotz immunmodulatorischem Potential liegen bisher keine Untersuchungen zu Galektin-2 in GDM vor. Ziel der hier vorgelegten Fall-Kontroll-Untersuchung (GDM vs. Kontrolle) war daher die systematische Charakterisierung der Galektin-2 Expression in maternalem und fetalem Plazentagewebe mittels Immunhistochemie und Doppelimmunfluoreszenz

(Publikation 1). Es fand sich eine signifikant erhöhte Galektin-2 Expression in GDM Plazenten.

Die Datenlage zu Histondysregulationen in GDM ist insbesondere in fetalen Proben unvollständig [86]. Acetylierung des 9. Lysins (H3K9ac) und Trimethylierung des 4. Lysins (H3K4me3) sind zwei „aktivierende" PTMs des Histon 3, die u.a. für die Regulation von Transkription, Zelldifferenzierung und Synzytium-Bildung eine Rolle spielen [94-99]. In der vorliegenden Studie (Publikation 2) wurde die globale Expression dieser Histonmodifikationen in Kontroll- und GDM Plazenten untersucht. Mittels Immunhistochemie und Doppelimmunfluoreszenz konnte eine signifikant verringerte H3K9ac Expression in GDM Plazenten gezeigt werden. In weiterführenden Zellkulturmodellen wurde außerdem die Wirkung von Vitamin D (Calcitriol) auf diese epigenetische Dysregulation untersucht. Hier zeigte sich eine Runterregulation der H3K9ac Produktion durch Vitamin D.

Übergreifendes Ziel der vorliegenden Studien war es, durch Charakterisierung plazentarer Proteinexpressionsmuster zu einem besseren Verständnis der Pathophysiologie des GDM beizutragen und eine Basis für die weiterführende Forschung an relevanten Signaltransduktionswegen zu schaffen.

1.4. Studiendesign & Methodik

Die Diagnose GDM wird durch einen Screeningtest am Ende des 2. Trimenons gestellt. Die Rekrutierung der Teilnehmerinnen dieser Fall-Kontroll-Studie erfolgte in der Spätschwangerschaft, nach Diagnosestellung. Eine nicht-invasive Beschaffung von Plazentagewebe, war daher erst nach Entbindung möglich.

1.4.1. Studienkollektiv

Nachdem das Studiendesign durch die Ethikkommission der LMU München genehmigt worden war, wurden 40 an GDM erkrankte und 40 gesunde Schwangere als Kohorten rekrutiert. Jede Patientin gab ihr schriftliches Einverständnis zur Teilnahme. Die Diagnose GDM wurde mittels 75-g-oGTT anhand der Diagnosekriterien der DDG und DGGG gestellt (Stand 2011) [100]. Die beiden Gruppen waren bzgl. des kindlichen Geschlechts ausgeglichen (je 20 männliche und weibliche Feten). Sie unterschieden sich signifikant bzgl. des kindlichen Geburtsgewichts (3611g vs. 3317g; $p = 0{,}019$) und mütterlichen BMI (25,6kg/m^2 vs. 22,3kg/m^2; $p < 0{,}001$). Andere klinische Kriterien (u.a. Gestationsalter, Alter der Mutter, Nabelschnur pH) zeigten keine signifikanten Unterschiede.

1.4.2. Immunhistochemie

Mittels Immunhistochemie können bestimmte Protein-Epitope an Gewebeschnitten mikroskopisch sichtbar gemacht werden. Die hierfür nötige Spezifität wird durch die Antigen-Antikörper (AK)-Bindung zwischen dem im Gewebe vorhandenen Epitop und einem ausgewählten Primärantikörper gewährleistet.
Nach Inkubation mit dem Primärantikörper erfolgt die „Sichtbarmachung". In den vorliegenden Studien wurde die Peroxidase-Anti-Peroxidase-(PAP) Methode als indirekte Nachweismethode angewandt, um diese Bindungen sichtbar zu machen. Hierbei wird ein Sekundärantikörper (Anti-Fab-AK), der direkt an eine Vielzahl von „Horseradish" Peroxidase (HRP) Enzyme gekoppelt ist, auf das Präparat gegeben. Nach Hinzugeben des Substrat(H_2O_2)-DAB-Chromogen-Gemischs läuft eine makroskopisch sichtbare Farbreaktion ab.

Die lichtmikroskopische Auswertung erfolgte durch Anwendung des „Immunoreactivity Score" (IRS). Dies ist ein in der Analyse immunhistochemischer Präparate etablierter, semi-quantitativer Score, der international eingesetzt wird [101]. Er setzt sich folgendermaßen zusammen:
Farbintensität (0: keine, 1: schwache, 2: moderate, 3: starke) x Prozentualer Anteil gefärbter Zellen (0: keine Zellen angefärbt, 1: <10%, 2: 10% - 50%, 3: 51% - 80%, 4: >80%)
Es ergeben sich Werte zwischen 0 und 12, welche ordinalskaliert sind. Die Untersucherabhängigkeit dieser Methode kann durch die Bewertung zweier unabhängiger Personen gemindert werden.

1.4.3. Immunfluoreszenz

Im Unterschied zur Immunhistochemie können bei der Immunfluoreszenz zwei Epitope gleichzeitig sichtbar gemacht werden. Die Spezifität des Nachweises ist ebenfalls durch die Primärantikörper-Bindung gegeben (oftmals dieselben). Der gleichzeitige Nachweis zweier Epitope basiert wiederum auf der Sekundärantikörperbindung. Die Sekundärantikörper sind jeweils an einen fluoreszierenden Farbstoff gebunden, welche sich in der Wellenlänge unterscheiden und daher als verschiedene Farben – klar getrennt, aber parallel – sichtbar sind. Der Nukleus kann zur besseren Orientierung durch einen dritten Farbstoff markiert werden (hier DAPI – 4',6-diamidino-2-phenylindole – blau).
Durch diese Methode kann der Phänotyp der exprimierenden Zellen zuverlässig verifiziert werden. In den vorliegenden Studien wurden anti-Cytokeratin-7-AK und anti-CD31-AK für die Identifikation extravillöser Throphoblastzellen und fetaler Endothelzellen eingesetzt [102, 103].

1.4.4. Western Blotting

Western Blotting dient dem Nachweis spezifischer Proteine oder Proteinmodifikationen aus einem Lysat (Zellen oder Gewebe) und deren Quantifizierung. In der vorliegenden Studie wurde dadurch der Einfluss von Vitamin D auf Produktion von H3K9ac (und FOXO1) quantifiziert.

Zunächst erfolgt eine Gelelektrophorese: Proteine werden mithilfe eines elektrischen Feldes anhand von Ladung und Molekulargewicht aufgetrennt. Das darauffolgende Blotten dient der Übertragung der aufgetrennten Proteine auf eine strukturell stabile Polyvenylidenfluorid (PVDF) Membran. Erst auf diesem Material kann das gesuchte Protein sichtbar gemacht werden. Die Übertragung erfolgt abermals durch die Anlage eines elektrischen Feldes, welches nun senkrecht zu dem vorherigen steht.

Wie in der Immunhistochemie basiert die Farbreaktion auf der Interaktion von spezifischen Primärantikörpern und substratgebundenen Sekundärantikörpern. Bei der Western Blot Färbung sind die Sekundärantikörper an Biotin gebunden, welches in Verbindung mit Chromogen-Substrat-Lösung, durch eine enzymatische Reaktion zum Farbausfall führt.

Für die quantitative Auswertung, wurde die PVDF Membran in der Bio-Rad Universal Hood II gescannt und anschließend mit Hilfe der Bio-Rad Quantity One® Software ausgewertet. Es wurde die Farbintensität pro mm^2 der einzelnen Banden gemessen. Dies ist repräsentativ für die Expression der jeweiligen Proteine. β-Aktin Banden dienten hierbei als endogene Kontrolle. Das heißt die H3K9ac- (bzw. FOXO1-) Farbintensität wurde als prozentuale Intensität der β-Aktin-Farbintensität dargestellt.

1.5. Beitrag zu den gelisteten Publikationen

Paula Hepp (P.H.) führte die in Publikation 1 veröffentlichten Experimente durch, wertete die relevanten Daten für Publikationen 1 & 2 aus und visualisierte diese. Für beide Publikationen verfasste P.H. den ersten Entwurf des Manuskripts (Einleitung, Ergebnisse, Diskussion und Methoden) und überarbeite diese in Zusammenarbeit mit den Ko-Autor*innen gemäß den Anforderungen der Reviewer. (Siehe auch Abschnitt „Authors' Contributions" der jeweiligen Publikation.)

2. ZUSAMMENFASSUNG

Gegenstand der vorliegenden Dissertation ist die Untersuchung inflammatorischer und epigenetischer plazentarer Dysregulationen bei Gestationsdiabetes (GDM).

Pathophysiologisch besteht beim GDM eine Insulinresistenz, ähnlich einem Diabetes Mellitus Typ 2, die von einer chronischen Inflammation begleitet wird [18, 46]. Zunehmend rücken auch epigenetische Dysregulationen und die damit einhergehend veränderte Genexpression in den Fokus der Forschung [84].

Bei den vorgelegten Publikationen handelt es sich um Fall-Kontroll-Studien, in welchen immunologisch bzw. epigenetisch relevante Proteine auf mögliche Dysregulationen bei GDM untersucht wurden.

Die erste Publikation beinhaltet die systematische Analyse der Galektin-2 Expression in GDM Plazenten und Kontrollen. Wie Galektine allgemein [53], zeigt auch Galektin-2 vielfältige immunomodulatorische Effekte – sowohl anti- als auch pro-inflammatorisch [70, 71]. Die Analyse erfolgte mittels Immunhistochemie und Doppelimmunfluoreszenz. Dies ermöglichte eine semi-quantitative und qualitative Auswertung. Es zeigte sich eine signifikant erhöhte Galectin-2 Expression in GDM Plazenten. Ausgehend von der aktuellen Studienlage sind zwei Hypothesen naheliegend: 1) Die erhöhte Galectin-2 Expression ist Folge chronischer Inflammation und die anti-inflammatorischen Effekte dienen der Kompensation. 2) Die pro-inflammatorischen Effekte von Galectin-2 tragen zur Pathophysiologie des GDM bei. Aufgrund der deskriptiven Natur der vorliegenden Studie sind weiterführende Schlussfolgerungen jedoch nicht möglich.

In der zweiten Publikation wurden zwei post-translationale Modifikationen (PTMs) des Histons 3 (Acetylierung des 9. Lysins (H3K9ac) und Trimethylierung des 4. Lysins (H3K4me3)) untersucht. PTMs der Histone sind wichtige Bausteine der Epigenetik [76]. H3K9ac und H3K4me3 sind „aktive“ Modifikationen und so z.B. für die Initiierung der Transkription entscheidend [94, 95, 97]. Die immunhistochemische Untersuchung zeigte eine signifikant verringerte Expression von H3K9ac in GDM Plazenten. Die H3K4me3 Expression war nicht verändert. Vitamin-D-Mangel ist ein Risikofaktor für die Entwicklung von GDM [23]. Daher wurde die Wirkung von Vitamin D auf H3K9ac in weiterführenden Zellkulturmodellen untersucht. Hier zeigte sich kein positiver Effekt. Die global verringerte H3K9ac Expression könnte mit einer herabgesetzten plazentaren Transkriptionsaktivität einhergehen [104-106] und Implikationen für die Langzeitkomplikationen der Kinder haben. Auch hier bedarf es weiterer funktionaler Untersuchungen.

Die vorliegenden Publikationen tragen zum weiteren Verständnis der Pathophysiologie des GDM bei. Dies kann hoffentlich als Ausgangspunkt für die notwendige weiterführende experimentelle, klinische und epidemiologische Forschung dienen.

3. SUMMARY (ZUSAMMENFASSUNG ENGLISCH)

The subject of this thesis is the analysis of inflammatory and epigenetic dysregulations in gestational diabetes (GDM) on the placental level.

Pathophysiologically GDM represents a state of insulin resistance, similar to diabetes mellitus type 2, which is associated with chronic subclinical inflammation [18, 46]. Increasingly research is also focusing on epigenetic dysregulations and the changes in gene expression that accompany them [84].

This thesis contains two case-control-studies, which investigate the expression patterns of proteins (or post-translational modifications (PTMs)), relevant for inflammatory and epigenetic pathways.

The first publication presents a systematic analysis of galectin-2 expression in GDM and control placentas. Galectin-2, as galectins in general [53], has diverse immunomodulatory effects – pro- as well as anti-inflammatory in nature [70, 71]. Immunohistochemistry and double immunofluorescence were used for the analysis of galectin-2 expression. This allowed for semi-quantitative and qualitative evaluation. Galectin-2 expression was significantly higher in GDM placentas compared to controls. Based on current research two hypotheses can be formulated: 1) The increased galectin-2 expression is a result of chronic inflammation and aims at counterbalancing via anti-inflammatory effects. 2) The pro-inflammatory effects of galectin-2 contribute to the pathology of GDM. However, due to the descriptive nature of the presented results, further conclusion cannot be drawn at this point.

The second publication investigates two PTMs of histone protein 3: acetylation of lysine 3 (H3K9ac) and trimethylation of lysine 4 (H3K4me3). Histone modifications are an important component of epigenetic signaling [76]. H3K9ac and H3K4me3 are “active” modifications, for instance playing an important role for the initiation of transcription [94, 95, 97]. Immunohistochemical analysis showed a significantly lower H3K9ac expression in GDM, while H3K4me3 expression was not altered. Vitamin D deficiency has been established as a risk factor for GDM [23]. Based on that, the effect of Vitamin D on H3K9ac was investigated further using cell culture models. This showed no positive effect. The globally reduced H3K9ac expression may be associated with reduced transcriptional activity of the placenta [104-106] and may have implications for the long-term health of the offspring. Again, further research is needed to better understand functional interactions.

These two publications contribute to the growing understanding of the pathophysiology of GDM, by characterizing placental protein expression and dysregulation in GDM. May this form the basis for further experimental, clinical and epidemiological research.

4. PUBLIKATIONEN

4.1. "PLACENTAL GALECTIN-2 EXPRESSION IN GESTATIONAL DIABETES: A SYSTEMATIC, HISTOLOGICAL ANALYSIS"

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Placental Galectin-2 Expression in Gestational Diabetes: A Systematic, Histological Analysis

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Abstract: Gestational diabetes mellitus (GDM) is the most common pregnancy-associated metabolic disorder that negatively impacts on the health of both mothers and their offspring in the long-term. The molecular mechanisms involved are not fully understood. As in other states of insulin resistance, a disproportionate immune response in GDM leads to a state of chronic low-grade inflammation. Galectin-2 exerts regulatory effects on different immune cells. This study investigated galectin-2 expression in the placenta of 40 GDM patients and 40 controls, in a sex-specific manner. Immunohistochemistry was used for semi-quantitative analysis of expression strength. The phenotypes of galectin-2 expressing cells were characterized through double immunofluorescence. We found a significant up-regulation of galectin-2 in the fetal syncytiotrophoblast, as well as in the maternal decidua of GDM placentas. Double staining showed a strong galectin-2 expression in extra villous trophoblast cells and fetal endothelial cells in GDM. These findings present the first systematic investigation of galectin-2 in GDM. The findings contribute to the emerging understanding of the role of immunomodulation and inflammation in GDM and of galectin-2 itself. This might also have implications for the long-term cardiovascular health of the offspring.

Keywords: galectin-2; gestational diabetes (GDM); placenta; insulin resistance; chronic low-grade inflammation; metabolism

1. Introduction

Galectin proteins are those members of the large lectin family, which by definition contain a specific amino-acid sequence—the so-called carbohydrate recognition domain (CRD)—and thus bind the β-galactoside sugar domains [1,2]. The ever-growing protein family is currently classified according to their molecular structure, more specifically the number of different CRDs—prototype galectins contain only one CRD and, therefore, tend to form dimers in order to fulfil cross-linking functions. Tandem repeat galectins contain two different CRDs, while the chimera-type galectin contain one CRD and one N-terminal non-CRD domain within one molecule [3].

Extensive research into the physiological significance and functioning of galectins has been conducted over the past decades. The binding of specific Gal(β1-4)GlcNAc or Gal(β1-3)GlcNAc-terminating oligosaccharides in the CRD and the following crosslinking, form one basis of galectin

action [4–6]. One specific galectin fulfils numerous functions that might not only be different but even contrary, depending on tissue type and intra- or extracellular location [1,7]. By now 16 different galectins have been identified in humans and the majority are expressed in placental tissue [8]. Other organ systems that show a strong galectin involvement include the intestine and immune cells [9,10]. Galectins are modulators of ubiquitous physiological pathways, such as cell migration, cell-to-cell communication, apoptosis, and proliferation [11,12]. Therefore, they play an important role in numerous more complex processes, most prominently angiogenesis, metabolism, and immunomodulation [10,13,14]. These in turn are of immense importance for pathologies, including cancer progression, cardiovascular disease, and of course innumerable inflammatory disorders—all of which had galectin dysregulations that have been linked with [15–18].

Angiogenesis and immunomodulation form the basis of many mechanisms in physiological pregnancy, including implantation, placentation, and feto-maternal tolerance. It is, therefore, not surprising that galectins have become a focus in the field of reproductive biology. Galectins-1 and -3 are best studied in this context. Using extensive mouse models Blois et al. [19] were able to show the pivotal role of galectin-1 in feto-maternal tolerance. Knockout resulted in higher rates of fetal loss, which could to a certain extend be reversed through treatment with recombinant galectin-1. The pathway through which galectin-1 acts are brought forward here includes the induction of tolerogenic dendritic cells and regulatory T-cells. These results were underpinned by further studies in humans [20]. A galectin-3 knock-out similarly hindered implantation in mouse models [21].

The pathophysiological connection between faulty implantation, inadequate immune response, and numerous pregnancy disorders is well-known and various galectin dysregulation are found in pathologies, including preeclampsia (PE), intrauterine growth restriction (IUGR), and HELLP-syndrome [22–24]. In PE, molecular research has come as far as identifying galectin-13 (also known as placental protein-13 (PP-13)) as a clinically relevant predictive marker for the development of PE [25].

Another pregnancy disorder showing galectin involvement is gestational diabetes mellitus (GDM). It is the most common metabolic-disorder in pregnancy and—contrary to most obstetric morbidities—its prevalence has steadily increased over the past decades [26–28]. It has grave implications in terms of perinatal complications, due to the associated risk of PE, fetal macrosomia, and C-sections [29,30]. The burden of GDM is further aggravated by the long-term morbidity it causes—mother and offspring stand a significantly higher risk of developing type 2 diabetes (DM2), adiposity, metabolic syndrome, and cardiovascular disease [31–35].

Similar to other pregnancy disorders, the pathophysiology of gestational diabetes involves the dysregulation of immunoregulation. GDM manifests when the anti-insulinemic effect of placental steroid hormones is not balanced with an increased maternal insulin production in beta-cells [36]. Furthermore, it was found that GDM resembles a state of insulin resistance similar to DM2 [37]. This is also accompanied by a chronic state of low-grade inflammation [38]; for example, interleukin (IL) 6 and tissue inhibitor of metalloprotease 1 (TIMP-1), a marker for low-grade inflammation, were shown to be elevated in GDM [39,40]. Remarkably, epidemiological studies were able to show a significant association between these markers and cardiovascular risk in the general population and a GDM cohort, respectively [41,42].

As modulators of inflammation, some members of the galectin family have previously been described in GDM. Results of these studies showed that galectin-1, -3, and -13 are significantly dysregulated in the placentas of GDM patients, and galectin-13 has recently been tested as a potential biomarker for GDM-screening [43–46].

Galectin-2, on the other hand, is generally not as well understood. It belongs to the prototype galectins, thus, contains only one CRD and typically forms a homo-dimer [2,47]. Like other members of the galectin family, it is involved in immunomodulation. Interestingly, it has been linked with pro- as well as anti-inflammatory actions [48,49]. Previous studies on placental tissue showed a significant decrease of galectin-2 in miscarriages, preeclampsia, and male IUGR cases [23,50,51].

To the best of our knowledge so far there are no reports on the role of galectin-2 in GDM and only few reports concerning other forms of diabetes are available. One large genetic–epidemiological study of 3,272 British women was conducted by Christensen et al. [52]. It showed that the functional A:T rs7291467 single nucleoid polymorphism (SNP), which in the galectin-2 gene (LGALS2) affects the gene's transcription level [53], is associated with higher fasting levels of insulin and glucose. Interestingly, the same SNP was found to be associated with a risk of cardiovascular disease in different Asian population [54,55], but no association was found in European population studies [56,57].

Considering what is currently known as GDM's pathophysiology and functions of galectins, we hypothesized, that galectin-2 expression is altered in GDM placentas.

This study presents a sex-specific, systematic analysis of galectin-2 expression in control and GDM placentas, as well as a characterization of galectin-2 expressing phenotypes. The aim of which is to establish whether galectin-2 dysregulation plays a part in the pathophysiology of GDM. This might be a starting point for further investigation into the role of galectin-2 in metabolic dysregulation.

We found galectin-2 to be significantly upregulated in both fetal syncytiotrophoblast (SCT) and maternal decidua of GDM placentas. The sex-specific analysis revealed no significant differences between female and male placentas.

2. Results

A systematic and sex-specific immunohistochemical and immunofluorescent analysis of galectin-2 expression was conducted on 40 healthy (20 female, 20 male foetuses) and 40 GDM (20 female, 20 male foetuses) placentas.

2.1. There Are No Sex-Specific Differences in Galectin-2 Expression

Since sex-specific differences are common in placental disorders, sex-disaggregated data collection was used throughout the study. Furthermore, statistical analysis was applied to check for sex-specific differences in galectin-2 expression, within the control group as well as the GDM group. However, no significant difference was found between male and female foetuses concerning the galectin-2 expression in either group. As can be seen in Figure 1, this was the case in fetal syncytiotrophoblast, as well as maternal decidual tissue.

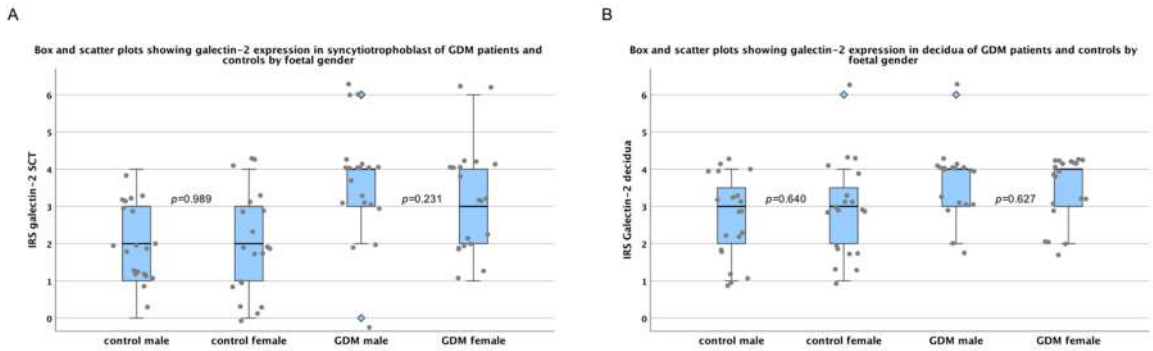

Figure 1. Sex-disaggregated galectin-2 expression. Box and scatter plots show the immunoreactivity score (IRS) for galectin-2 expression by fetal gender in syncytiotrophoblast (SCT; **A**) and decidua (**B**). The range between the 25th and 75th percentiles is represented by the boxes, with the horizontal line showing median. The bars indicate the 5th and 95th percentiles. Blue diamonds indicate values more than 1.5-times the boxes' lengths. Grey dots represent singular data points. There was no statistically significant difference between the male and female groups, as shown by the *p*-values.

2.2. Galectin-2 Expression Is Upregulated in the Fetal Syncytiotrophoblast of GDM Placentas

Based on the first statistical analysis of sex-specific differences described above, we pooled female and male controls and GDM placentas for the following analyses. In the fetal syncytiotrophoblasts, the immunohistochemical evaluation detected a low (IRS: 2) expression of galectin-2 in control placentas.

As can be seen in Figure 2, this expression was significantly upregulated in the SCT of GDM placentas ($p < 0.001$), with the median IRS coming to 4.

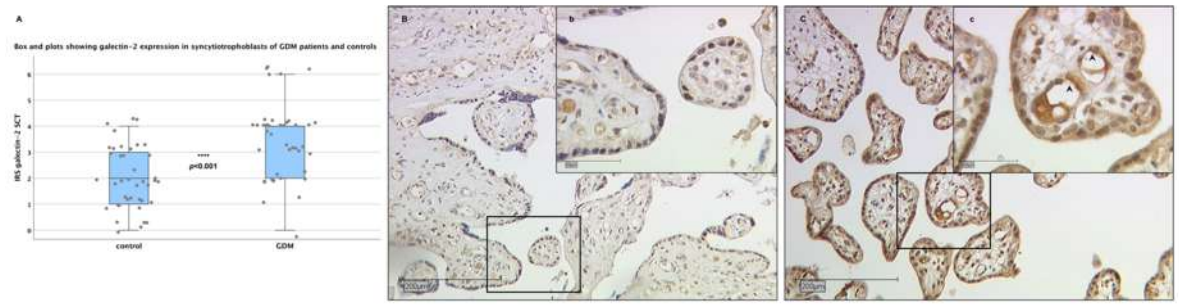

Figure 2. Galectin-2 expression in syncytiotrophoblast (SCT) of control and gestational diabetes mellitus (GDM) placentas. Box and scatter plots (**A**) showing the IRS for galectin-2 expression in SCT to be significantly higher in GDM placentas ($p < 0.001$). See Figure 1 for a detailed plot explanation. Pictures show representative slides for immunohistochemical staining of galectin-2 in the SCT of the control (**B**) and GDM (**C**) placentas. The scale bar equals 200 µm in full size images and 50µm in inserts. Black rectangles indicate the section seen at higher magnification (minuscular letters). Arrow heads show strongly stained fetal endothelial cells.

2.3. Galectin-2 Expression Is Upregulated in the Maternal Decidua of GDM Placentas

A similar pattern was found in the maternal decidua. The galectin-2 expression in the control group was low to moderate, with a median IRS of 3. Like the fetal syncytiotrophoblast, the maternal decidual tissue of the GDM group showed a significantly higher galectin-2 expression than the controls ($p = 0.01$; median IRS: 4; see Figure 3).

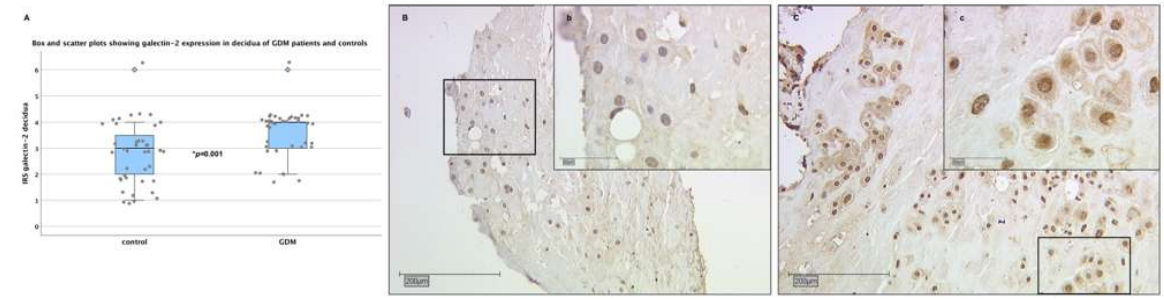

Figure 3. Galectin-2 expression in the maternal decidua of control and GDM placentas. Box and scatter plots (**A**) showing the IRS for galectin-2 expression in decidua to be significantly higher in GDM placentas ($p = 0.001$). See Figure 1 for a detailed box plot explanation. Pictures show representative slides for immunohistochemical staining of galectin-2, in the maternal decidua of the control (**B**) and the GDM (**C**) placentas. The scale bar equals 200 µm in full size images and 50µm in inserts. Black rectangles indicate the section seen at a higher magnification (minuscular letters).

2.4. There are No Significant Differences in Galectin-2 Expression between Normal and Overweight Pregnancies

Due to the fact that maternal body mass index (BMI), prior to pregnancies was significantly higher in the GDM group compared to controls, we conducted statistical analysis to investigate the influence of BMI on placental galecetin-2 expression. Our testing showed no significant difference between galectin-2 expression in subjects with low and normal BMI (<25kg/m^2) and in overweight and obese (BMI ≥25kg/m^2) patients. This was the case in fetal SCT ($p = 0.082$), as well maternal decidua ($p = 0.132$) tissue (Figure 4). Therefore, BMI did not seem to be a confounder of the differences in placental galectin-2 expression found in this study.

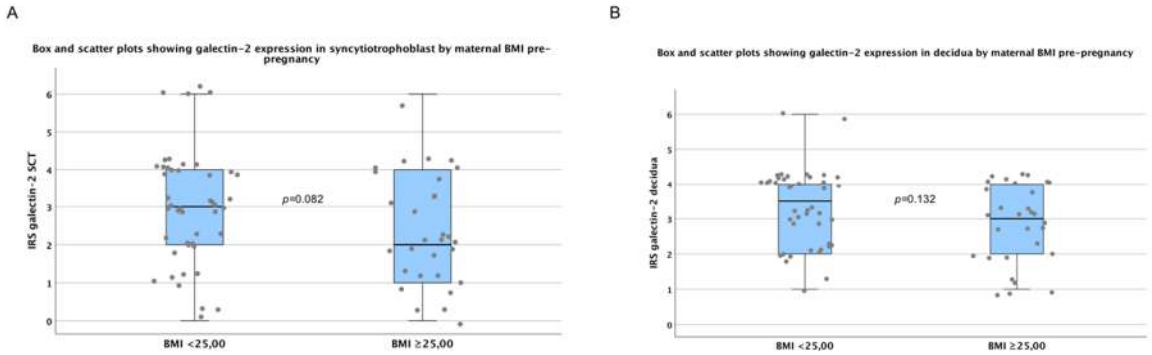

Figure 4. Galectin-2 expression by maternal body mass index (BMI) pre-pregnancy. There was no significant difference between the groups. For detailed plot explanation see Figure 1.

2.5. Identification of Galectin-2 Expressing Cells by Immunofluorescence Double Staining

In order to identify the phenotype of galectin-2 expressing cells, immunofluorescence double staining followed by fluorescent microscopy were carried out (see Figures 5 and 6; for magnified sections, see Figure S1). Cytokeratin 7 (CK7) and cluster of differentiation (CD) 31 were used as markers for the extra-villous trophoblast cells (EVTs) and fetal endothelial cells, respectively [58,59].

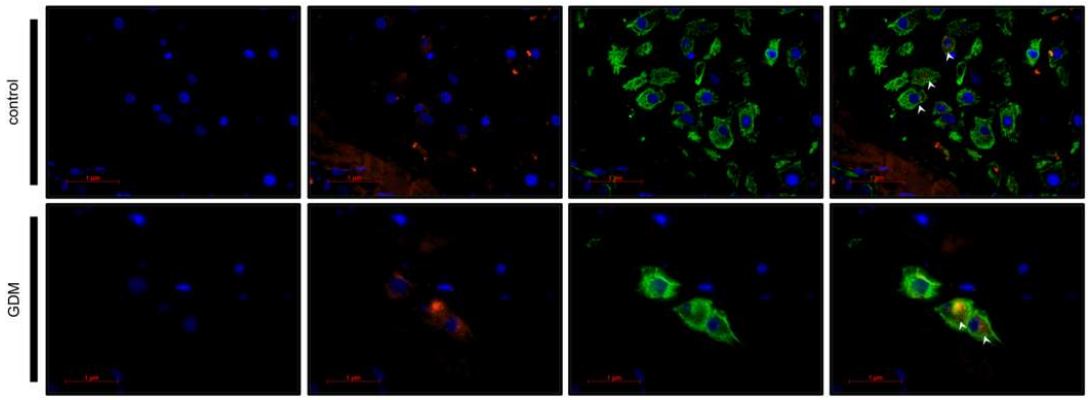

Figure 5. Double immunofluorescence phenotyping of decidual cell. Nuclei are stained blue using DAPI. Galectin-2, bound by Cy-3-labled secondary antibody, is stained red. CK7, bound by Cy-2-labeled secondary antibody is stained green, marking the extra-villous trophoblast (EVT) cells. Arrows heads indicate the merging expression of CK7 and galectin-2 visible as yellow. The scale bar equals 1 μm.

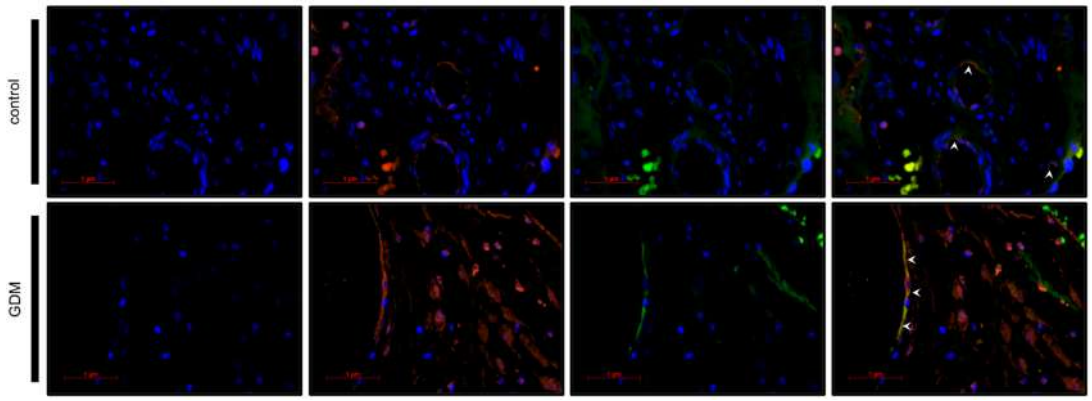

Figure 6. Double immunofluorescence phenotyping of villus cells. Nuclei are stained blue using DAPI. Galectin-2, bound by Cy-3-labled secondary antibody, is stained red. CD31, bound by Cy-2-labeled secondary antibody is stained green, marking fetal endothelial cells. Arrows heads indicate the merging expression of CD31 and galectin-2, visible as yellow. The scale bar equals 1 μm.

Microscopic evaluation confirmed CK7 and galectin-2 expression in the same cell, thereby, identifying fetal EVTs as the predominant galectin-2 expressing cell type in the maternal decidua of the GDM, as well as the control group (see Table 1). The galectin-2 expression in GDM placentas appeared to be more intense, compared to the control.

Table 1. Quantification of galectin-2 expressing cells.

	Number of Galectin-2 Positive Cells Evaluated	Galectin-2 and CK7 Double Positive Cells	Percentage of Galectin-2 and CK7 Positive Cells
Control	41	39	95,1 %
GDM	79	77	97,5 %

Similarly, fluorescence microscopy demonstrated the co-expression of CD31 and galectin-2, thus, verifying galectin-2 expression by endothelial cells. Again galectin-2 expression appeared to be more intense in GDM. There were also numerous CD31 negative cells in the villus mesoderm of GDM cases that expressed galectin-2.

3. Discussion

Galectins are major players in the regulation of immunomodulation and metabolism and their dysregulation takes up an important role in numerous pathologies, including GDM. Our study corroborates the current evidence by showing an increased expression of galectin-2 in the syncytiotrophoblast, EVT, and the fetal endothelial cells of GDM patients. This was the first investigation to establish a role of galectin-2 dysregulation in the pathophysiology of GDM.

Blois et al. [44] previously showed a dysregulation of galectin-1 in GDM patients. While peripheral galectin-1 serum levels in GDM patients were lower compared to controls, expression in the placenta was significantly increased. Since galectin-1 is generally considered to be anti-inflammatory, it was hypothesized that its upregulation might be a reaction to the state of chronic inflammation present in GDM, aiming to restore balance [10,11,44].

Both galectin-1 and galectin-2 are prototype galectins and showed a 47% structural overlap in humans. However, in terms of T-cell interaction, the two galectins bind distinct glycoproteins and are, hence, thought to induce different cellular pathways [60]. Therefore, it should not be assumed that their involvement in immunoregulation could be seen as one. Moreover, a large number of studies have been conducted on galectin-1's receptor binding and transcription modulation in trophoblast cells, while this kind of information is still largely lacking for galectin-2 [8].

Nevertheless, information on the galectin-2 function is accumulating through investigations in models of auto-immune diseases. In a model of auto-immune dermatitis, Loser et al. [61] showed a comparable upregulation of galectin-2 in the affected skin tissue. In the same study treatment, galectin-2 reduced the number of activated CD8+ T-cells while the regulatory T-cells (CD4+) were not affected [61]. Similarly, galectin-2 shifted T-cell cytokine profiles towards a T-helper-cell (Th) 2 phenotype downregulation of interferon (INF) γ, tumor necrosis factor (TNF) α, and upregulation of IL-5 [60]. Based on these findings, further investigations into the therapeutic potential of galectin-2 were conducted; these showed a significant reduction of inflammation in acute and chronic mice colitis-models [48]. In activated neutrophils, galectin-2 induced externalization of phosphatidylserine, leading to phagocytosis [62]. In light of these anti-inflammatory qualities, it seemed reasonable to consider the increased placental expression of galectin-2 as a reaction to the state of chronic inflammation present in GDM.

Very recently, Maeda et al. [63] were the first to show that galectin-2 involvement is direct in adequate insulin secretion of pancreatic beta-cells. In their study, disruption of galectin-2 resulted in reduced insulin secretion. Should galectin-2 also be upregulated systemically in GDM cases, it might be

hypothesized that this upregulation aims at stabilizing insulin secretion in beta-cells. Therefore, further investigations into galectin-2 on protein and DNA level in mothers as well as offspring are necessary.

On the other hand, Yildirim et al. [49] recently showed pro-inflammatory effects of galectin-2 via polarization of M0 macrophages into a proinflammatory M1 state, which is accompanied by increased concentrations of inflammatory cytokines, including IL-6 and TNFa. This clarified that immunomodulation through galectins is extremely complex and the mechanisms induced even by one member of the galectin family vary, depending on the cell type [64].

In normal pregnancy, macrophages account for around 20% of decidual leukocytes. Typically, a shift from M1 to M2 phenotype can be observed on a cellular as well as epigenetic level [65]. Therefore, a second hypothesis emerges. The increased galectin-2 expression in GDM characterized in this study might contribute to the proinflammatory milieu of insulin resistance via macrophage dysregulation. Further studies on macrophage phenotype and cytokine profiles in GDM should be conducted to gain further insights into the complexity of galectin-2 function in this context.

Nonetheless, this complexity seems to somewhat mirror the complexity of alterations in cytokine profile in GDM mothers recently characterized by Ategbo et al. [66]. Both an increase of proinflammatory IL-6 and TNFα and the downregulation of Th1/Th2 ratio described in their paper, could hypothetically be mediated (at least partially) by an increased galectin-2 expression. It must be noted, however, that the cytokine and T-cell profile in macrocosmic offspring differed greatly—a fact that yet needs to be clarified [66].

Through double immunofluorescence we were able to show that the upregulation of galectin-2 is not restricted to the trophoblast, but directly affects fetal endothelial cells, as well. Thus, implications for angiogenesis and cardiovascular disease should also be considered.

An induction of the M1 phenotype (as described above) goes hand in hand with reduced numbers of M2 macrophages, which are important producers of pro-arteriogenic factors like vascular endothelial growth factor (VEGF) A or matrix metalloproteinase (MMP) 2 [49]. This might be a causal link through which galectin-2 exerts its anti-arteriogenic effect, previously described in patients with coronary heart disease (CHD) and CHD mouse models [67,68]. More broadly, cardiovascular diseases are generally linked to inflammation and increased levels of IL-6 were shown to be significantly associated with a risk of CHD [41].

Taking into consideration the strong expression of galectin-2 in fetal endothelial cells and surrounding CD31-negative cells of GDM placentas (see Figure 5), this might well be relevant for the development of life-long cardiovascular risk of the offspring. Hypothetically, an increased concentration of galectin-2 could lead to a perivascular M1 phenotype induction, which in turn could lead to a pro-inflammatory state that interferes with adequate vascularization.

In conclusion, the present study establishes the role of galectin-2 dysregulation in the pathophysiology of GDM. Due to the descriptive nature of our findings the question arises whether the increased galectin-2 expression is a reaction to the inflammatory state of GDM or whether it might contribute to its development. Considering current knowledge on galectin-2 function, either conclusion seems somewhat reasonable. Hence, further research is urgently needed to clarify its role in GDM and potentially reveal its therapeutic implications.

4. Materials and Methods

4.1. Tissue Samples

After the study design was approved by the LMU ethics committee, 40 GDM patients and 40 healthy expectant mothers (control) were chosen to participate. Fetal gender was balanced in both groups. Written consent was obtained from all participants in advance. To be included in the study, all participants underwent an oral glucose tolerance test (oGTT) between week 24 and 28 of their pregnancy. The diagnosis of GDM was based on the criteria of the German society for Diabetes Mellitus (two measurements above limits—fasting glucose >90 mg/dL, 1 h > 180 mg/dL, and 2 h > 155 mg/dL) [69]. Clinical and epidemiological data of the study cohort is depicted in Tables 2 and 3.

Table 2. Clinical and epidemiological data of study cohort by fetal gender. The data were analysed using the Kruskal Wallis Test.

	GDM		Control		*p*-Value
	Male	Female	Male	Female	
Birthweight (g)	3662.1 ± 562	3635.9 ± 661	3339.8 ± 568	3294 ± 440	$p = 0.019$ *
Duration of gestation at delivery (weeks)	39.67 ± 1.30	39.83 ± 1.40	39.80 ± 1.54	39.75 ± 1.16	$p = 0.943$
Maternal BMI pre-pregnancy (kg/m^2)	29.38 ± 8.03	26.96 ± 4.73	21.92 ± 3.97	25.04 ± 7.90	$p < 0.001$ *
pH in umbilical artery	7.30 ± 0.07	7.30 ± 0.10	7.28 ± 0.10	7.30 ± 0.08	$p = 0.826$
Maternal Age (years)	31.46 ± 4.12	33.21 ± 5.33	30.30 ± 6.11	32.00 ± 6.13	$p = 0.177$

Statistically significant differences are marked with an asterisk (*).

Table 3. Number of GDM and control subjects per BMI group [70].

	Number of Patients in GDM Group	Number of Patients in Control Group
Underweight (BMI < 18.5 kg/m^2)	0	4
Normal BMI (18.5–24.9 kg/m^2)	16	25
Overweight (25.0–29.9 kg/m^2)	10	3
Obese (≥30.0 kg/m^2)	12	5

Tissue samples ($2 \times 2 \times 2$ cm^3) from a central cotyledon of the participants' placentas were obtained directly after birth. The areas of sampling contained maternal decidua, fetal syncytiotrophoblast, and amniotic epithelia. Macroscopically they were sufficiently supplied with blood, while areas with signs of calcification, bleeding, or ischemia were avoided. After 24 h of fixation in 4% buffered formalin solution, the tissue samples were embedded in paraffin for long-term storage.

4.2. Immunohistochemistry

4.2.1. Staining

The immunohistochemical staining was based on a detailed protocol recently published by Hutter et al. [71]. A general overview is given in the following. After removal of paraffin in a Roticlear (Carl Roth, Karlsruhe, Germany) bath, endogenous peroxidase activity was blocked using 3% H_2O_2. Second, the protein epitopes were demasked by high-pressure sodium citrate (pH 6.0) treatment. Blocking solution (ZytoChem Plus HRP Polymer System, Zytomed Systems GmBH, Berlin, Germany) was applied to prevent unspecific antigen–antibody interaction. Thereafter, the slides were incubated with primary antibodies—anti-galectin-2-antibody (polyclonal rabbit IgG, concentration 0.05mg/mL, NBP1-89690, Novus Biologicals, Minneapolis, USA) dissolved in PBS at 1:200 dilution for 16 h, at 4 °C. After washing the slides with PBS they were treated with Post Block (Reagent 2, ZytoChem Plus HRP Polymer System mouse/rabbit, Zytomed) for 20 min, followed by HRP Polymer (Reagent 3, ZytoChem Plus HRP Polymer System mouse/rabbit, Zytomed) for 30 min. Visualization was achieved by applying chromogen 3,3'–diaminoenzidine (DAB; Dako, Glostrup, Denmark). Positive and negative control staining was carried out on human colon tissue, alongside each round of staining (see Figure 7). Positive controls served to ensure viability of the antibody. Negative controls were used to rule out any unspecific staining. In order to achieve this negative control, serum (negative control for super sensitive rabbit antibodies, rabbit IgG, Biogenics, Fremont, USA) containing anti-rabbit-Igs was applied instead of the primary antibody. Mayer's hemalum was used for counterstaining. After dehydration in an ascending series of alcohol and Roticlear (Carl Roth, Karlsruhe, Germany) treatment, the slides were cover slipped.

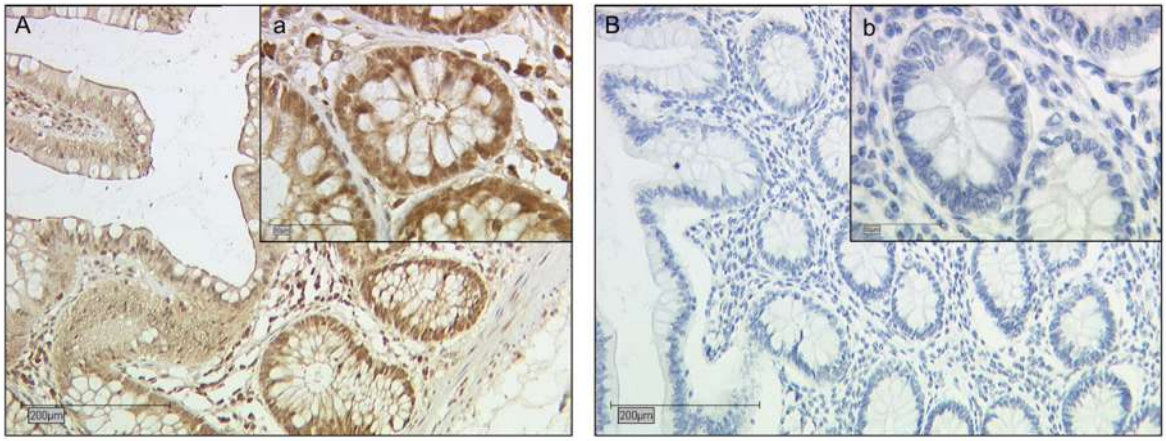

Figure 7. Immunohistochemistry control staining. Colon tissue was used as positive (**A**, **a**) as well as negative (**B**, **b**) control, for anti-galectin-2-antibody.

4.2.2. Evaluation

All samples were evaluated under a Leitz Diaplan microscope using 10-fold and 40-fold objectives (see also corresponding photographs in result section). The semi-quantitative Immunoreactivity Score (IRS) [72] was used to evaluate tissue staining—cell staining intensity (0: none; 1: weak; 2: moderate; 3: strong) and the percentage of positively stained cells (0: no staining; 1: <10% of the cells; 2: 11–50%; 3: 51–80%; 4: >80) were evaluated separately and, thereafter, the values were multiplied, resulting in an IRS between 0 and 12 for each slide. One slide of high staining quality was evaluated per participant on which 3–5 microscopic fields and a minimum of 100 cells were counted. All slides were evaluated by two independent observers, with no more than two sessions per observer. If the observers came to diverging conclusions, the sample was re-evaluated and discussed until reaching one conclusive result.

4.3. Double Immunofluorescence

For the phenotypical characterization of galectin-2 expressing cells double immunofluorescence was conducted with CK7 as a marker for extra villous trophoblast cells [58] and CD31 as a marker for fetal endothelial cells [59].

After removal of paraffin and de-masking of all protein epitopes (see Immunohistochemistry), blocking solution (Ultra V–Block, Thermo Scientific, Lab Vision, Fremont, CA, USA) was applied for 15 min, in order to prevent unspecific antigen–antibody interaction. The slides were then incubated with the primary antibody mixtures (see Table 4 for details). Subsequently the fluorescent secondary antibodies were applied for 30 min (see Table 4 for details). Following the antibody incubation, the slides were cover slipped using mounting buffer (Vector Laboratories, Burlingame USA), which contains DAPI for nuclear counterstaining. Light exposure was kept to a minimum during the covering process. The fluorescent Axioskop photomicroscope (Zeiss, Oberkochen, Germany) was used for evaluation of fluorescent staining. Photographs were taken with a digital Axiocam camera system (Zeiss, Oberkochen, Germany). Evaluation and documentation were done under a 63-fold objective. For quantification of the relative share of EVT cells of the galectin-2 expression, the number of galectin-2 expressing cells, and that of CK7-galectin-2 double positive cells were counted on the generated pictures.

Table 4. Antibody features used for double-immunofluorescence.

Antibody	Dilution	Incubation	Manufacturer
Galectin-2—polyclonal Rabbit IgG	1:200	16 h at 4 °C	Novus Biologicals—NBP1-89690
CK7—Clone OVTL Mouse IgG	1:30	16 h at 4 °C	Novocastra—NCL-L-CK7-OVTL
CD31—Clone JC/70A Mouse IgG	1:50	16 h at 4 °C	Abcam—ab9498
Cy-2-labelled goat-anti-rabbit	1:100	30 min at RT	Dianova—115-226-062
Cy-3-labelled goat-anti-mouse	1:500	30 min at RT	Dianova—111-165-144

4.4. Statistical Analysis

IBM SPPS Statistics (Version 22.0. for Windows, Armonk, NY, USA) was used for data collection, analysis, and visualization. The non-parametric Man-Whitney-U test was used for the analysis of statistical significance, which was assumed to be at $p < 0.05$.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/7/2404/s1.

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Abbreviations

BMI	Body Mass Index
CD	Cluster of differentiation
CHD	Coronary heart disease
CK7	Cytokeratin 7
CRD	Carbohydrate recognition domain
DM2	Diabetes Mellitus Type 2
EVT	Extra-villous trophoblast cells
g	Gramm
GDM	Gestational Diabetes Mellitus
h	Hours
IL	Interleukin
INF	Interferon
IRS	Immunoreactivity score
IUGR	Intra uterine growth restriction
MMP	Matrix metalloproteinase
oGTT	Oral glucose tolerance test

PE	Preeclampsia
PP-13	Placental protein 13
SCT	Syncytiotrophoblast
Th	T-helper-cell
TIMP-1	Tissue inhibitor of metalloprotease 1
TNF	Tumor necrosis factor
VEGF-A	Vascular endothelial growth factor A

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4.2. "Histone H3 Lysine 9 Acetylation is Downregulated in GDM Placentas and Calcitriol Supplementation Enhanced This Effect"

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Histone H3 Lysine 9 Acetylation is Downregulated in GDM Placentas and Calcitriol Supplementation Enhanced This Effect

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Abstract: Despite the ever-rising incidence of Gestational Diabetes Mellitus (GDM) and its implications for long-term health of mothers and offspring, the underlying molecular mechanisms remain to be elucidated. To contribute to this, the present study's objectives are to conduct a sex-specific analysis of active histone modifications in placentas affected by GDM and to investigate the effect of calcitriol on trophoblast cell's transcriptional status. The expression of Histone H3 lysine 9 acetylation (H3K9ac) and Histone H3 lysine 4 trimethylation (H3K4me3) was evaluated in 40 control and 40 GDM (20 male and 20 female each) placentas using immunohistochemistry and immunofluorescence. The choriocarcinoma cell line BeWo and primary human villous trophoblast cells were treated with calcitriol (48 h). Thereafter, western blots were used to quantify concentrations of H3K9ac and the transcription factor FOXO1. H3K9ac expression was downregulated in GDM placentas, while H3K4me3 expression was not significantly different. Cell culture experiments showed a slight downregulation of H3K9ac after calcitriol stimulation at the highest concentration. FOXO1 expression showed a dose-dependent increase. Our data supports previous research suggesting that epigenetic dysregulations play a key role in gestational diabetes mellitus. Insufficient transcriptional activity may be part of its pathophysiology and this cannot be rescued by calcitriol.

Keywords: histone modification; H3K9ac; H3K4me3; FOXO1; epigenetics; gestational diabetes; vitamin D

1. Introduction

Gestational Diabetes Mellitus (GDM) is defined as glucose intolerance firstly detected during pregnancy [1]. It is the most common pregnancy-related metabolic disorder, affecting up to 14% of pregnancies [2]. Contrary to other obstetric complications, its prevalence has increased steadily over the past decades, making it an urgent issue in pre- and perinatal care [3,4].

The short-term consequences of GDM include a higher risk for preeclampsia (Odds Ratio (OR) 1.81), large for gestational age infants (LGA) (OR 3.43) and C-section (OR 1.46) [5,6]. However, GDM also affects long-term health outcomes of both mothers and offspring. Women who suffered from GDM stand a substantially higher risk of developing diabetes mellitus type 2 (DM2) later in life (relative risk (RR) 7.43) [7]. Their offspring also suffer from a higher risk for developing metabolic syndrome, DM2, adiposity and cardiovascular disease [8–10].

GDM manifests when the increased need for insulin during pregnancy cannot be met, leading to a state of glucose intolerance, insulin resistance and inflammation similar to DM2 [11,12]. As the producer of anti-insulin hormones and in its function as the foeto-maternal-interface, the placenta is thought to play a key role in the pathophysiology of GDM in general and, more specifically, in the long-term health programming of the offspring [13,14]. However, despite the urgent need for effective treatment and disease prevention in future generations, the pathophysiology's underlying molecular mechanisms are not yet fully understood.

Increasingly, pathophysiological research focuses on transcriptional and epigenetic mechanisms of insulin resistance. Evidence constantly emerges that transcription factors, including FOXO1, co-factors and chromatin modifications, like post-translational histone modifications, interact on various levels of gene expression and through this, contribute to insulin resistance. These mechanisms may also be part of the developmental programming of metabolic diseases [15].

Barker's "Developmental Origins of Human Health and Disease" theory, stating that foetal environment influences health outcomes in later life, first arose from investigations of the relationship between birthweight and cardiovascular diseases in adulthood [16,17]. Since then, results from various cell culture and animal models, as well as epidemiological investigations point towards epigenetics as being one of the underlying molecular mechanisms [18–21].

Epigenetics can be seen as "the sum of the alterations to the chromatin template that collectively establish and propagate different patterns of gene expression and silencing from the same genome" [22]. The three main mechanisms interacting to form the epigenome are: DNA methylation, histone modifications and non-coding RNA (ncRNA) [22]. While evidence of epigenetic changes in GDM cases is growing, research has been focusing mainly on DNA methylation studies, with data on histone modifications in GDM is still widely lacking [23].

Post-translational Modification (PTM) of histone proteins influences transcription, DNA replication and other processes via cis and/or trans mechanisms. The direct cis mechanism describes a change of inter-nucleosome contact, thus affecting the chromatin condensation state. Histone acetylation is known to have a vast potency for unfolding chromatin. The indirect trans mechanism refers to the recruitment of "reader" proteins to the chromatin, which in turn recruit further modifying proteins. Additionally, to cis and trans interactions, interaction between different PTMs, so called "cross-talk", also occur [22,24,25]. As the "cross-talk" interactions indicate, the sum of histone modifications results in a histone-code, which determines the accessibility of a given chromatin section.

The two modifications acetylation of histone 3 at lysine 9 (H3K9ac) and trimethylation of histone 3 at lysine 4 (H3K4me3) are known to be "active" modifications, and their presence at Transcriptional Start Site (TSS) regions correlates with the amplitude of gene expression [26]. High levels of H3K4me3 can be found specifically at the promotor region of active genes, because it plays a role in forming the preinitiation complex [27,28]. H3K9ac enrichment, on the other hand, marks active enhancers. Furthermore, both PTMs are found at bivalent promoters marking regulatory genes of importance for cell differentiation [26,29]. These findings indicate that both H3K9ac and H3K4me3, like epigenetics in general, are of great importance for an organism's development and human disease. Their role in GDM, however, has not yet been investigated.

FOXO1 is part of the Forkhead box protein subfamily O, a group of transcription factors containing winged helix DNA-binding domain [30]. In its function as a transcription factor, it plays a key role in metabolic regulation, especially in the glucose metabolism. Increased FOXO1 has been shown to stimulate gluconeogenesis as well as lipid production in liver cells [31,32]. Reduced FOXO1 function on the other hand was shown to protect against insulin resistance under a high fat diet in mice [33]. More recent results were able to show that FOXO1 is elevated in GDM placentas on mRNA as well protein level. Furthermore, placental *FOXO1* mRNA expression was significantly correlated with the HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) in the GDM group [34]. Further studies with vitamin D receptor (VDR) knockout mice extrapolated a functional link between vitamin D deficiency, elevated FOXO1 and insulin resistance in muscle cells [35].

The epidemiological connection between vitamin D deficiency and GDM has been discussed, but remains controversial, with several studies suggesting that there is a positive correlation between low vitamin D and insulin resistance or GDM respectively [36–38], while others did not find a significant connection [39–41]. Recent metanalyses suggest, however, that vitamin D deficiency is in fact a risk factor (OR) for the development of GDM [42,43]. Investigations on GDM placentas have shown an increased expression of VDR compared to control placentas and hypothesised this to be a reaction to low vitamin D levels [44]. Recently, ChiP-seq analyses in cell culture experiments by Meyer, et al. [45] showed that stimulation with Calcitriol ($1,25(OH)_2D_3$) lead to an enrichment of H3K9ac at promotor regions of VDR regulated genes, suggesting a link between epigenetic modifications and vitamin D.

The aim of this study was to conduct a systematic, sex-specific immunohistochemical and immunofluorescence analysis of active histone modifications in GDM placentas. Furthermore, cell culture experiments were carried out to test the hypothesis that vitamin D stimulation could increase H3K9ac and correspondingly decrease FOXO1 expression in the choriocarcinoma cell line BeWo. Cell culture results were verified using primary human villous trophoblast (HVT) cultures.

We found H3K9ac to be upregulated in syncytiotrophoblast, extra villous trophoblast (EVT), as well as foetal endothelial cells in GDM placentas. The analysis of H3K4me3 did not reveal any significant differences in expression between GDM and control placentas. The stimulation of BeWo cells with human calcitriol resulted in a decrease of H3K9ac at high concentrations and no significant changes at low concentrations. This corresponded with an increase in FOXO1 expression after stimulation. This indicates that the dysregulated H3K9ac expression in GDM cannot be salvaged by vitamin D.

2. Results

The expression of specific post-translational modifications of histone protein 3 were analysed in placental tissue from 40 heathy (20 of female, 20 of male foetuses) and 40 GDM pregnancies (20 of female, 20 of male foetuses) using immunohistochemistry and double immunofluorescence. The modifications under investigation were H3 lysine 9 acetylation (H3K9ac) and H3 lysine 4 trimethylation (H3K4me3).

2.1. H3K9ac Expression is Downregulated in GDM Placentas

There was no sex-specific difference of H3K9ac expression within the control group, nor within the GDM group. Therefore, we grouped male and female data of control and GDM group for the following data analysis. A very strong H3K9ac expression (median immuno reactive score (IRS): 12) was detected in nuclei of villous syncytiothrophoblast cells (SCT), extra villous trophoblast cells (EVT) as well as foetal endothelial cells in control placentas. The boxplot in Figure 1A illustrates that the expression of H3K9ac was significantly downregulated in the syncytiotrophoblast cells of GDM placentas ($p < 0.001$), with a median IRS of 12 in the control compared to 8 in the GDM placentas. The same pattern was found in EVTs (Figure 1D–F) and foetal endothelial cells (Figure 1G–I), where statistical analysis proved the downregulation to be highly significant ($p < 0.001$).

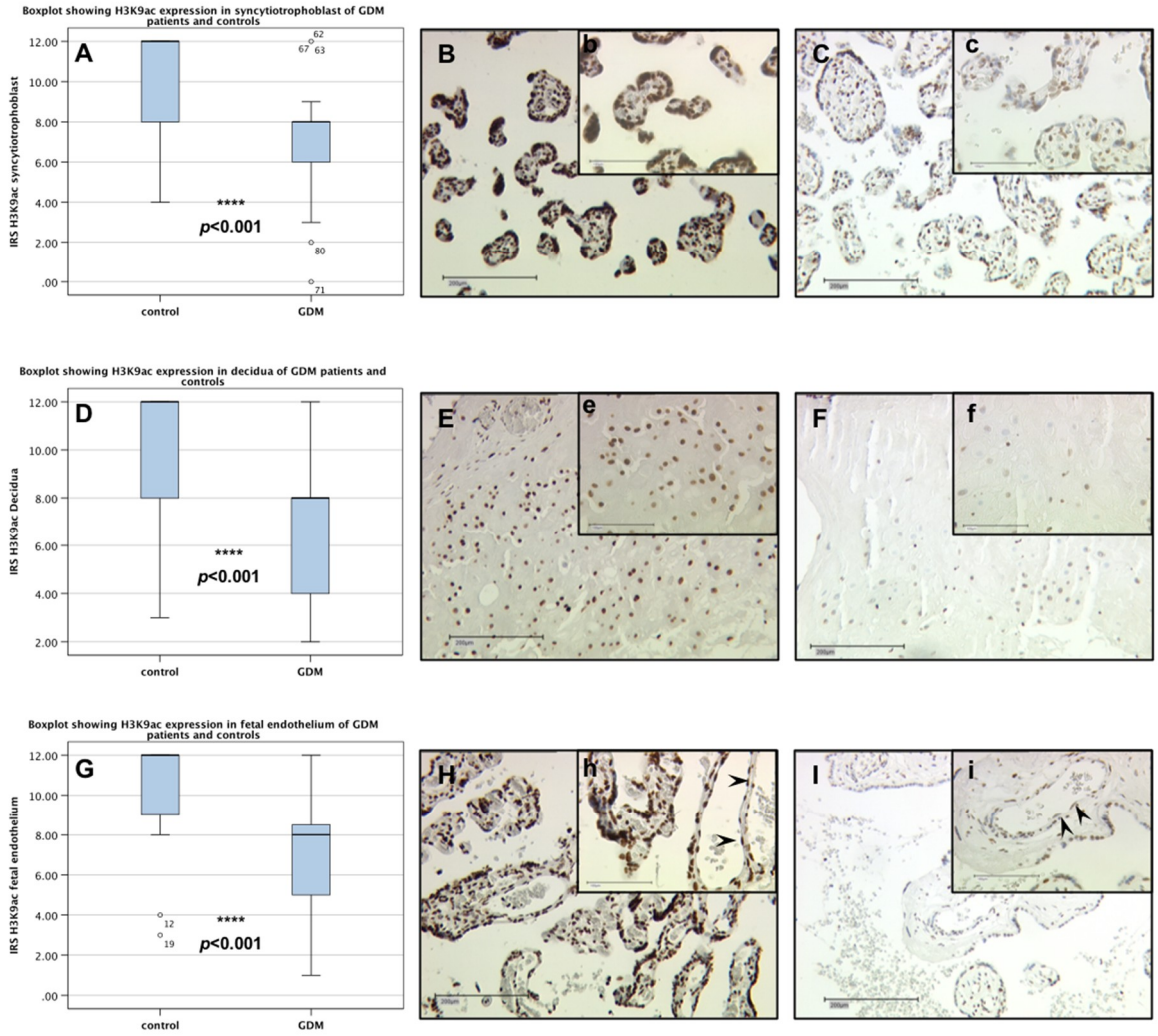

Figure 1. H3K9ac expression in syncytiotrophoblast, decidua and foetal endothelia cells of control and GDM placentas. Boxplots (**A**,**D**,**G**) showing the IRS for H3K9ac expression in syncytiotrophoblast (SCT), decidua and foetal endothelial to be highly significantly lower in GDM placentas ($p < 0.001$). The range between the 25th and 75th percentiles is represented by the boxes with the horizontal line showing median. The bars indicate the 5th and 95th percentiles. Circles indicate values more than 1.5-times the boxes' lengths. Pictures showing representative slides for immunohistochemical staining of H3K9ac in the SCT (control (**B**,**b**); GDM (**C**,**c**)), decidua (control (**E**,**e**); GDM (**F**,**f**)) and foetal endothelial cells (control (**H**,**h**); GDM (**I**,**i**)) of according placentas. Arrowheads indicate foetal endothelial cells. Pictures were taken with a 100× lens (capital letters) and 250× lens (lower-case letters) respectively.

2.2. No Difference between H3K4me3 Expression in GDM and Control Placentas

In contrast to H3K9ac expression, no statistically significant differences were found in the expression of H3K4me3 (see Figure S1). The median IRS for H3K4me3 expression in the syncytiotrophoblast was 8 in control, as well as GDM placentas ($p = 0.853$). Similar results were obtained for EVTs. There was however a significant difference in the H3K4me3 expression between male and female control placentas ($p = 0.040$).

2.3. Identification of H3K9ac Expressing Cells by Immunofluorescence Double Staining

Immunofluorescence double staining was carried out using Cytokeratin 7 (CK7) and Cluster of differentiation 31 (CD31) as identifying markers for EVTs and foetal endothelial cells respectively. By using triple filter excitation microscopy, cell phenotypes could be identified (Figure 2).

Double filter excitation showed CK7 and H3K9ac expression in the same cell, thus confirming the expression of H3K9ac by EVTs. H3K9ac was also expressed by some CK7-negative decidual stroma cells. The H3K9ac expression was more intense in EVT cells of control placentas than GDM placentas. Similarly, double filter excitation showed co-expression of CD31 and H3K9ac, confirming H3K9ac expression by endothelial cells. Again, the H3K9ac expression was more intense in endothelial cells of control placentas than GDM placentas.

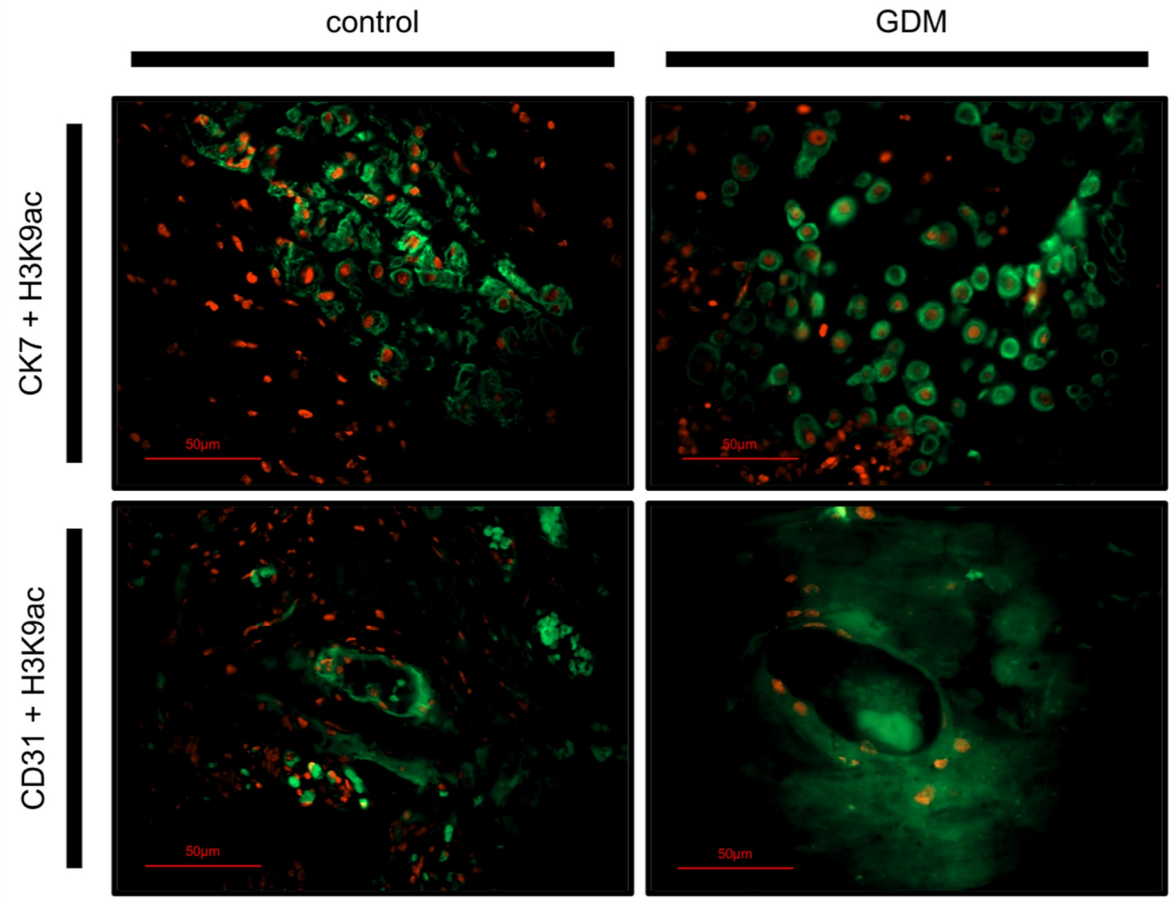

Figure 2. Double immunofluorescence phenotyping of placenta cells. H3K9ac, marked with Cy-3-labled secondary antibody, is stained red in both rows. CK7 is stained green in the first row, marking EVT cells. CD31 is stained green in the second row, marking endothelial cells. Pictures were taken with a 400× lens.

2.4. Downregulation of H3K9ac in Trophoblast Tumour Cells BeWo by Human Calcitriol (Vitamin D)

To gain insight into the possible functional relationship between H3K9ac and FOXO1 expression and human calcitriol (Vit. D) Western Blot analysis was used to quantified H3K9ac expression in BeWo cells after Vit. D stimulation.

Quantitative Western Blot analysis showed that the 48 h in vitro culture with 1.0 µM human calcitriol resulted in a significantly lower H3K9ac expression in BeWo cells (Figure 3B,C; $p = 0.008$). However, the stimulation with lower concentrations of human calcitriol (0.1 and 0.01 µM), though lowering H3K9ac expression slightly, did not have a significant influence on the expression of H3K9ac ($p = 0.515$ for both concentrations).

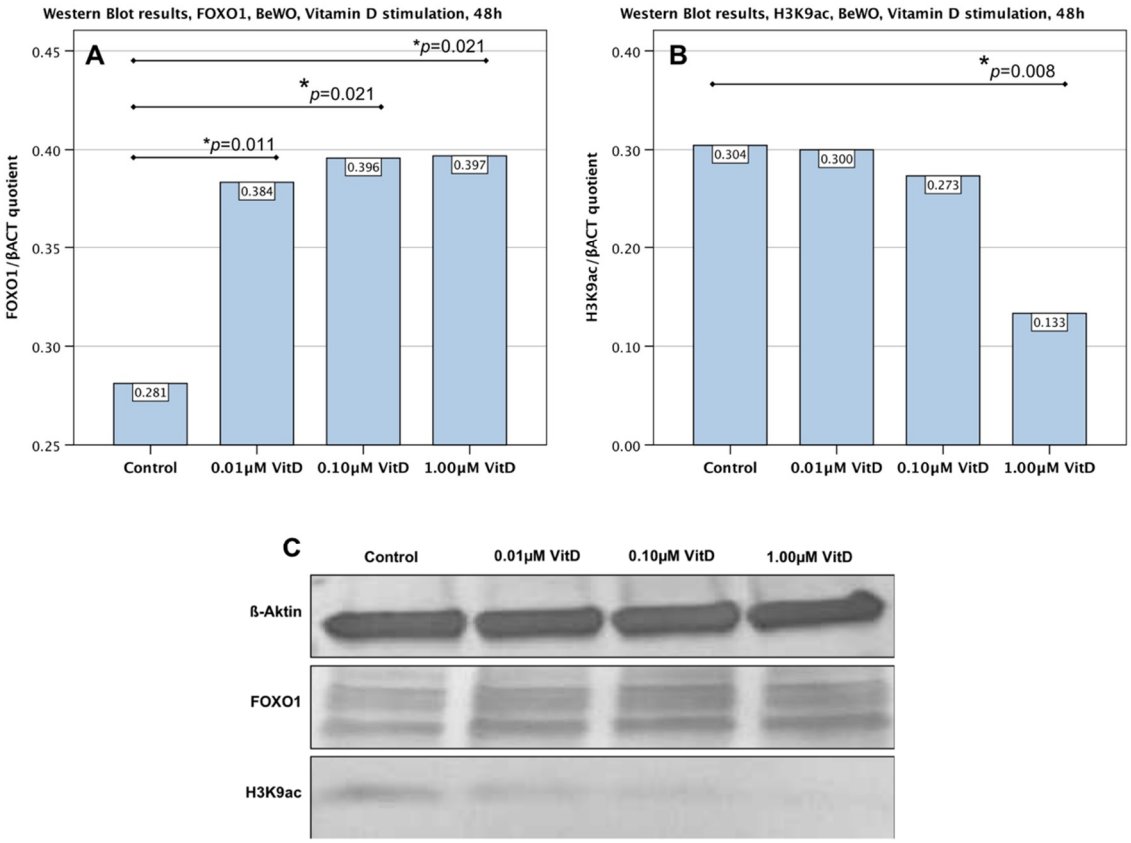

Figure 3. Western Blot analysis of FOXO1 and H3K9ac expression in BeWo cells after 48 h stimulation with human calcitriol. (**A**) Bar graph diagram showing a significant (p = 0.011 corresponding to 0.01 μM, p = 0.021 corresponding to 0.1 μM, p = 0.021 corresponding to 1.0 μM), dose-dependent upregulation of FOXO1 after 48 h stimulation with human calcitriol. (**B**) Bar graph diagram showing a significant (p = 0.008) downregulation of H3K9ac after 48 h stimulation with human calcitriol at 1.0 μM. No significance was shown for stimulation with 0.01 nor 0.1 μM. (**C**) Representative photographs of the western blot membrane with detected bands for H3K9, FOXO1 and endogenous control (β-Actin).

2.5. Upregulation of FOXO1 in Trophoblast Tumour Cells BeWo by Human Calcitriol (Vitamin D)

The expression of FOXO1 in BeWo cells after vitamin D stimulation was found to increase in a dose-dependent manner (Figure 3A,C). Statistical analysis showed the increased expression to be significant (p = 0.011) even after stimulation with the lowest concentration (0.01 μM). FOXO1 expression increased further with increasing concentrations of the stimulant, still showing statistical significance (p = 0.021).

2.6. Downregulation of H3K9ac in Primary Human Villous Trophoblast Cells HVT by Human Calcitriol (Vitamin D)

To solidify results concerning the downregulation of H3K9ac in BeWo cells, the experiment was repeated using human villous trophoblast cells. The stimulation of primary culture human villous trophoblast cells (HVT) with 1.0 μM human calcitriol showed an effect similar to BeWo cells. As seen in Figure 4, the expression of H3K9ac was significantly decreased (p = 0.03) after a 48 h incubation in comparison with the control culture.

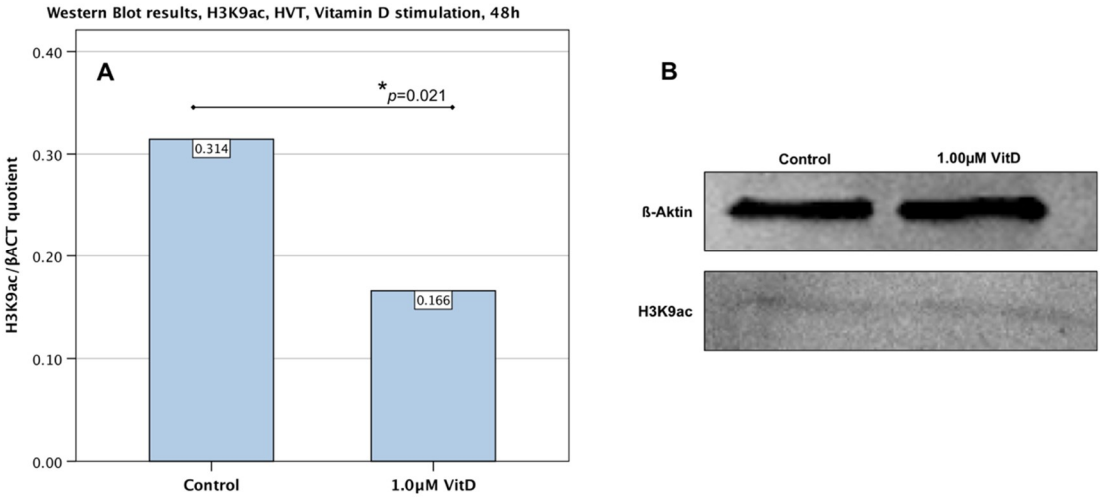

Figure 4. Western Blot analysis of H3K9ac expression in HVT cells after 48 h stimulation with human calcitriol. (**A**) Bar graph diagram showing a significant (p = 0.03) downregulation of H3K9ac after 48 h stimulation with human calcitriol at 1.0 µM. (**B**) Representative photograph of the western blot membrane with detected bands for H3K9 and endogenous control (β-Actin).

3. Discussion

Dysregulation of histone modifications is an important factor in the pathophysiology of metabolic diseases and foetal programming, including GDM. The present study provides further evidence of this, as we identified a significant downregulation of H3K9ac in syncytiotrophoblast, EVT and foetal endothelial cells in GDM cases. Additional investigations of H3K4me3 did not show any dysregulation in GDM placentas.

While studies on insulin resistance were able to identify changes in histone modifications on a gene-specific level [46,47], this is the first study to show a pan-placental downregulation of H3K9ac in gestational diabetes mellitus. H3K9ac is an important modification for transcription activity in general and especially relevant for intrauterine development, synzytialisation and angiogenesis [26,48,49]. Animal studies have shown that treatment of somatic cell nuclear transfer embryos with histone deacetylase (HDAC)-inhibitors and the corresponding increase in global H3K9ac levels lead to improved embryo development and blastocyst quality [50]. Thus, the global H3K9ac downregulation found in GDM may indicate insufficient capacity of gene expression. This reduction in transcriptional activity could in turn be linked to foetal complications such as organ immaturity.

By now it has been well established that pregnancy-related diseases—including GDM—show sex-specific differences in terms of pathophysiology and outcome [51,52]. Very recently, Alexander, et al. [53] were able to show sex-specific differences in GDM on an epigenetic level. Thus, we considered a sex-disaggregated collection of data to be of great importance for the present study. The analysis of our data, however, did not show any sex-specific differences in H3K9ac expression.

Research investigating the "cross-talk" between H3K9ac and H3K4me3 indicates that acetylation of H3 tails is dependent on the recognition of and binding to H3K4me3. Thus, H3K4me3 could be seen as a docking basis for further activation of chromatin through H3 acetylation [54]. These findings correspond with the PTM's localizations. As mentioned before, H3K4me3 is an essential part of the pre-initiation complex, forming the basis of transcription start, with transcription factor IID (TFIID) selectively binding to H3K4me3. H3K9ac is part of active enhancers, and as such potentiates transcription activity [55]. Taking this into account our findings may suggest that, while the basis for gene transcription is still intact, the activation of chromatin is in fact impaired in GDM placentas.

Investigating the role of histone modifications in insulin resistance, studies on beta-cells showed that treatment with incretin hormones, substances commonly used in treatment of DM2, leads to a global increase in H3K9ac. This in turn leads to an increase in cAMP response element binding

protein/CREB regulated transcription coactivator 2 (CREB/CRTC2) transcription factor activity [56], indicating the functional relevance of H3K9ac. Since incretin generally improves insulin sensitivity, these findings suggest that low H3K9ac levels may contribute to insulin resistance.

As outlined in the introduction, epidemiological as well as biochemical findings suggest a functional relationship between vitamin D, H3K9ac and FOXO1 with potential relevance for GDM. Thus, we hypothesized that treatment with vitamin D will positively affect H3K9ac (upregulate) and FOXO1 (downregulate). This hypothesis was tested through cell culture experiments using BeWo cells as a trophoblast model and HVT primary cultures to confirm (only H3K9ac). However, the results refuted our hypothesis. H3K9ac expression was not affected by low doses of calcitriol and decreased slightly at the highest concentration. FOXO1 expression increased after stimulation with calcitriol. While these results are consistent within each other, they resemble a state of glucose resistance rather than an improvement. Here it is important to note that stimulation of C2C12 muscle cells with calcitriol did result in a downregulation of FOXO1, suggesting that its role in metabolic control is highly cell type-specific [35]. We have summarized the immunohistochemical and cell culture findings graphically in Figure 5.

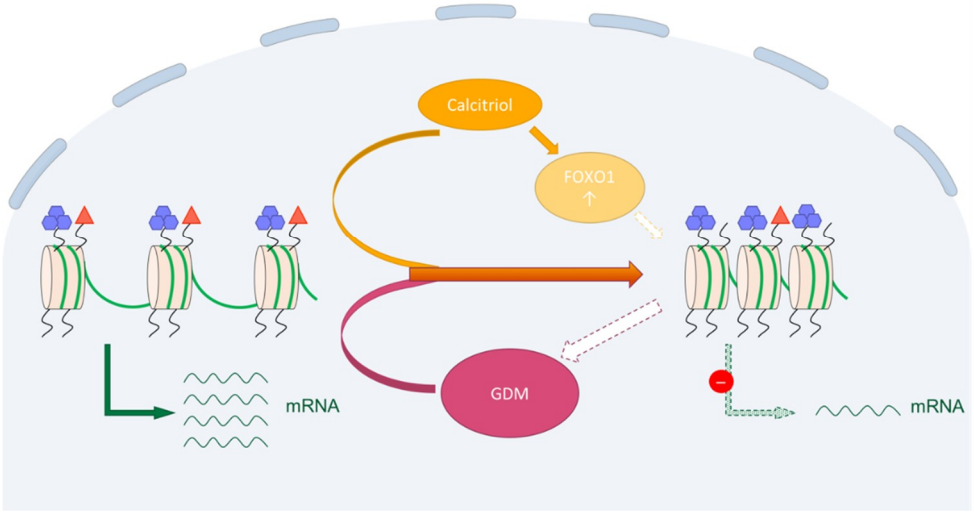

Figure 5. Two potential mechanisms leading to a reduction of H3K9ac in trophoblast cells. Immunohistochemical analysis showed a downregulation of H3K9ac (red triangle) in GDM placentas, while H3K4me3 (purple hexagons) showed no significant differences (solid pink arrow tail). The H3K9ac reduction may also be part of the aetiology of GDM (pink arrow broken line). Stimulation of trophoblast cells with calcitriol also lead to a reduction of H3K9ac, as well as an increase in FOXO1 (solid orange arrows). This may in turn be linked to the reduction of H3K9ac (yellow arrow broken line), ultimately leading to a downregulation of transcription (green arrows).

Extensive cell culture experiments using a dexamethasone as well as a TNF-induced model of insulin resistance were able to show that both agents induced VDR expression. Furthermore, they were able to show that VDR overexpression resulted in a reduction of insulin-mediated glucose uptake. These findings suggest that VDR is itself a mediator of different pathways of insulin resistance [57]. This may explain why treatment of BeWo cells with calcitriol, leading to an increased VDR activity, was unable to salvage H3K9ac and FOXO1 expression profiles, but rather mimicked a state of GDM.

The fact that we found a dysregulation of H3K9ac not only in SCT and EVT, but also in foetal endothelial cells, may indicate long-lasting effects for the offspring in general and their vascular system in particular. There is evidence that even short-term exposure to hyperglycaemia induces lasting epigenetic changes in vascular cells [58]. These epigenetic changes were causally linked to changes in gene expression, leading to an inflammatory and proatherogenic state [59]. Taking this into account we hypothesis that the downregulation of H3K9ac in foetal endothelial cells may contribute to the

foetal programming of cardiovascular disease associated with GDM. The role of H3K9ac in "metabolic memory" of the vascular system is controversial. Chen, et al. [60] showed an increase of non-specific H3 acetylation in human umbilical vein endothelial cells after glucose exposure. Furthermore, they found that the overexpression of p300, a histone acetyltransferase (HAT), results in similar expression profiles (e.g., transcription of VEGF-A and fibronectin) as glucose exposure. A global downregulation of H3K9ac was, however, found in endothelium and, in general, in a state of hypoxia [61,62], which in GDM may interact with the state of hyperglycaemia. Thus, further research is needed, concentrating on functional pathways associated with the global downregulation of H3K9ac.

In conclusion, our findings corroborate the growing evidence that epigenetic dysregulations play a key role in gestational diabetes mellitus. Due to the tremendous complexity of epigenetic mechanisms, conclusions concerning functional implications are to be drawn with extreme caution. The pan-placental downregulation of H3K9ac in GDM cases may, however, reflect a downregulation of transcriptional activity. Whether this is cause or effect of the metabolic disorder needs to be investigated further. Our cell culture experiments suggest that treatment with vitamin D is not sufficient to rescue the epigenetic and transcriptional changes in GDM, making this another area where further research is needed urgently.

4. Materials and Methods

4.1. Tissue Samples

The study design was approved by the Ludwig Maximilians University's ethics committee and written consent was obtained from all participating patients. All participants underwent an oral glucose tolerance test (oGTT) between weeks 24 and 28 of their pregnancy. Using the criteria of the German society for Diabetes Mellitus (capillary whole blood; fasting glucose >90 mg/dL, 1 h > 180 mg/dL, and 2 h > 155 mg/dL) the diagnosis GDM was given when two measurements were above limits. For the study design, 40 patients with GDM and 40 healthy patients (control) were chosen to participate. In each group, foetal gender was balanced. For detailed information on clinical and epidemiological data see Table 1. All GDM patients underwent insulin treatment and were monitored at least once a week at the Diabetes Centre of the Department of Internal Medicine LMU Munich. The study was approved by the ethical committee of the University of Munich (project identification code: 337-06) on the 26th of August 2013 and informed consent was obtained from each patient in written form. Samples and clinical information were anonymized for statistical workup.

Tissue samples ($2 \times 2 \times 2$ cm^3) of the participants' placentas were obtain directly after birth. The pieces were taken from a cotyledon located the central part of placenta, with sufficient blood supply, aiming to contain decidua, villous as well as extra villous trophoblasts and amniotic epithelia. Areas showing signs of calcification, bleeding or ischemia were excluded from tissue collection. A 24 h incubation period in 4% buffered formalin solution served to fixate the tissue samples, after which they were embedded in paraffin for long-term storage.

Table 1. Clinical and epidemiological data of study participants. The Kruskal–Wallis and Man-Whitney-U Tests were used for analysis of the data.

	GDM		Control		*p*-Value
	Male	Female	Male	Female	
Birthweight (g)	3662.1 ± 562	3635.9 ± 661	3339.8 ± 568	3294 ± 440	$p = 0.019$ *
Duration of gestation at delivery (weeks)	39.67 ± 1.30	39.83 ± 1.40	39.80 ± 1.54	39.75 ± 1.16	$p = 0.943$
Maternal BMI (pre-pregnancy)	29.38 ± 8.03	26.96 ± 4.73	21.92 ± 3.97	25.04 ± 7.90	$p < 0.001$ *
pH in umbilical artery	7.30 ± 0.07	7.30 ± 0.10	7.28 ± 0.10	7.30 ± 0.08	$p = 0.826$
Maternal Age (years)	31.46 ± 4.12	33.21 ± 5.33	30.30 ± 6.11	32.00 ± 6.13	$p = 0.177$
Vaginal delivery (%)	50	75	80	80	$p = 0.207$

Significant differences are marked with an asterisk (*).

4.2. Immunohistochemistry

4.2.1. Staining

Immunohistochemical staining was carried out in accordance with the recently published protocol by Hutter, et al. [63]. After blocking the endogenous peroxidase using 3% H_2O_2, the slides were treated with sodium citrate (pH 6.0) in a high-pressure cooker in order to de-mask all protein epitopes. To prevent any unspecific antigen-antibody interaction, blocking solution was applied (ZytoChem Plus HRP Polymer System, Zytomed Systems GmBH, Berlin, Germany). The slides were then incubated with the primary antibodies (see Table 2) for 16 h at 4 °C. Antigen-antibody interaction was detected by applying the ZytoChem Plus HRP Polymer System (Zytomed Systems GmBH, Berlin, Germany) in accordance with the manufacturer's instructions. Chromogen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) was used for staining followed by haemalaun for counterstaining. After dehydration, the slides were cover-slipped. For each experiment, positive and negative control staining was carried out on human colon tissue (Figure 6).

Table 2. Antibodies applied for immunohistochemistry and double-immunofluorescence.

Antibody	Dilution	Incubation	Manufacturer
H3K4me3—polyclonal Rabbit IgG	1:500	16 h at 4 °C	Abcam—ab8580 (Cambridge, UK)
H3K9ac—Clone Y28 Rabbit IgG	1:200	16 h at 4 °C	Abcam—ab32129 (Cambridge, UK)
CK7—Clone OVTL Mouse IgG	1:30	16 h at 4 °C	Novocastra—NCL-L-CK7-OVTL (Newcastle, UK)
CD31—Clone JC/70A Mouse IgG	1:50	16 h at 4 °C	Abcam—ab9498 (Cambridge, UK)
Cy-2-labelled goat-anti-rabbit	1:100	30 min at RT	Dianova—115-226-062 (Hamburg, Germany)
Cy-3-labelled goat-anti-mouse	1:500	30 min at RT	Dianova—111-165-144 (Hamburg, Germany)

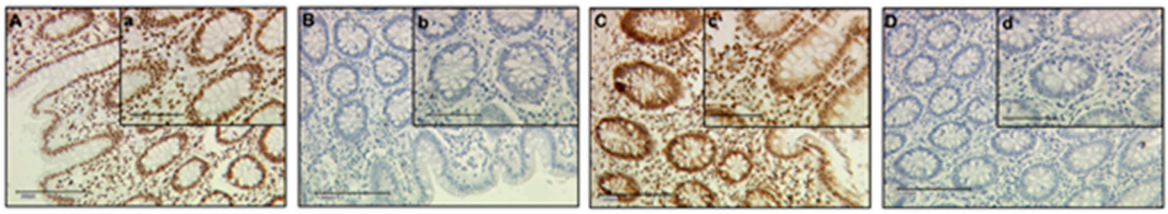

Figure 6. Positive and negative immunohistochemistry staining controls. Colon tissue was used as positive (**A**,**a**) as well as negative (**B**,**b**) control for H3K9ac antibody. For the H3K4me3 antibody, colon tissue was used as positive (**C**,**c**) as well as negative (**D**,**d**) control, as well. Negative control serum was added to the tissue for negative control staining. Brown staining indicates primary and secondary antibody binding, blue staining is due to haemalaun counter staining. Pictures were taken at a 100× (capital letters) and 250× (lower-case letters) magnification, respectively.

4.2.2. Evaluation

Analysis of the tissue samples was conducted under a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). The semi-quantitative *Immunoreactivity* Score (IRS) [64] was used to evaluate tissue staining. Multiplication of cell staining intensity (0: none; 1: weak; 2: moderate; 3: strong) with the percentage of positively stained cells (0: no staining; 1: <10% of the cells; 2: 11–50%; 3: 51–80%; 4: >81%) results in an IRS between 0 and 12 for each slide.

4.3. Double Immunofluorescence

To determine the phenotype of H3K9ac expressing cells in the placenta tissue, double immunofluorescence staining was performed. Placenta tissue of both the control and the GDM group were stained, using CK7 as a marker for EVTs and CD31 as a marker for foetal endothelial cells.

Tissue samples from the same patient collective were used for double immunofluorescence staining as for immunohistochemistry. Pre-treatment of the slides (deparaffinising, blocking of endogenous peroxidase activity, de-masking of protein epitopes) was identical to that used for immunohistochemistry. Blocking solution (Ultra V–Block, Thermo Scientific, Lab Vision, Fremont, CA, USA) was applied for 15 min in order to prevent any unspecific antigen-antibody binding. Thereafter, the primary antibodies were mixed and applied together (see Table 2). Following this, the slides were incubated with the secondary antibodies (see Table 2) for 30 min. The slides were then cover-slipped with minimal light exposure using mounting buffer (Vector Laboratories, Burlingame, CA, USA), which contains DAPI for nuclear counterstaining. The phenotypes were analysed using the fluorescent Axioskop photomicroscope (Zeiss, Oberkochen, Germany). Images were taken with a digital Axiocam camera system (Zeiss, Oberkochen, German).

4.4. Cell Culture and Stimulation

The choriocarcinoma cell line BeWo (ECACC, Salisbury, UK) was used as a trophoblast model. Human villous trophoblast cells (HVT) (ScienCell, Carlsbad, CA, USA), a primary cell culture which was kryoconserved at −196 °C, was used to confirm BeWo results. Both cell lines were cultured in DMEM (3.7 g/L $NaHCO_3$, 4.5 g/L D-glucose, 1.028 g/L stable glutamine, and Na-Pyruvate; Biochrom, Berlin, Germany) enriched with 10% foetal bovine serum (FBS) at 37 °C and 5% CO_2. The BeWo as well as the HVT cells were grown in a 12-well plate at a density of 500,000 cells/mL DMEM for western blotting. To ensure adherence the cells were firstly cultured in DMEM with 10% FBS for 4 h, after which it was replaced with fresh DMEM not containing any supplementation. Following the 24 h incubation, the cells were stimulated with 0.01, 0.1 and 1.0 µM of human calcitriol (Sigma-Aldrich, St. Louis, MO, USA), dissolved in ethanol and diluted in DMEM without FBS. Corresponding amounts of ethanol were added to the control cells (see Table 3 for stimulation scheme). Stimulation lasted 48 h.

Table 3. Cell culture stimulation scheme.

BeWo Stimulation for Western Blot (WB)			
1.0 µM Ethanol	0.01 µM Vit. D	0.1 µM Vit. D	1.0 µM Vit. D
HVT Stimulation for WB			
1.0 µM Ethanol		1.0 µM Vit. D	

4.5. Westernblotting of Stimulated BeWo Cells and HVT Cell

The cells were treated with 200 µL of lysis buffer for 30 min, consisting of RIPA (Radioimmunoprecipitation assay buffer, Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) (dilution 1:100). The obtained lysates were centrifuged. To determine the protein concentration a Bradford assay was carried out. The amount of protein chosen for β-Actin and H3K9ac/FOXO1 detection were 5 and 20–25 µg respectively. Firstly, the samples' proteins were separated by molecular weight through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and thereafter transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Blocking of unspecific background staining was performed by incubating the membrane in 1× Casein solution (VECTASTAIN ABC-AmP Kit for rabbit IgG, Vector Laboratories, Burlingame, CA, USA) for 1 h. The primary antibodies, anti-H3K9ac (monoclonal rabbit IgG, Abcam, Cambridge, UK), anti-FOXO1 (monoclonal mouse IgG, Novus Biologicals Europe, Abingdon, UK) diluted at 1:500 in Casein and anti-β-Actin (monoclonal mouse IgG, Sigma-Aldrich, St. Louise, MO, USA) diluted at 1:1000 in Casein, were added for 16 h at 4 °C, plus an additional 2 × 15 min incubation at room temperature for anti-H3K9ac and anti-FOXO1. Following washing in 1× Casein solution the membrane was incubated with the respective secondary antibodies, biotinylated anti-rabbit-/mouse-IgG (VECTASTAIN ABC-AmP Kit for rabbit/mouse IgG, Vector Laboratories, Burlingame, MA, USA) as instructed by manufacturer's manual. After 20 min treatment with

ABC-AmP-reagent (VECTASTAIN ABC-AmP Kit for rabbit IgG, Vector Laboratories, Burlingame, MA, USA), the blots were developed with 5-bromo-4-chloro-3′-indolyphosphate/nitro-blue tetrazolium (BCIP/NBT)-chromogen substrate solution (VECTASTAIN ABC-AmP Kit for rabbit IgG, Vector Laboratories, Burlingame, MA, USA). Blots were detected using the Bio-Rad Universal Hood II (Bio-Rad Laboratories, Hercules, CA, USA) and quantitative analysis was performed using Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

4.6. Statistical Analysis

IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA: IBM Corp was used for data collection, data analysis and charts. More specifically, the non-parametric Mann-Whitney-U test was used for comparison of IRS results and the Wilcoxon-signed rank test for analysis of western blot results. P-values smaller than 0.05 were considered statistically significant.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/19/12/4061/s1. Figure S1: Sex-disaggregated H3K4me3 expression in syncytiotrophoblast (**A**) and decidua (**B**) of control and GDM placentas.

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

Abbreviations

BMI	Body Mass Index
CD31	Cluster of differentiation 31
CK7	Cytokeratin 7
CREB/CRTC2	cAMP responsive element binding protein 1/CREB regulated transcriptional coactivator 2
DM2	Diabetes Mellitus Type 2
DMEM	Dulbecco's modified Eagle's medium
EVT	Extra-villous trophoblast cells
FBS	Fetal bovine serum
FOXO1	Forkhead box protein O1
g	Gramm
GDM	Gestational Diabetes Mellitus
h	Hours
H3K4me3	Trimethylation of Histone 3 at lysine 4
H3K9ac	Acetylation of Histone 3 at lysine 9
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HOMA-IR	Homeostatic model assessment—insulin resistance
HVT	Human villous trophoblast cells
IRS	Immunoreactivity score
LGA	Large for gestational age
M	Molar
mRNA	Messanger ribonuclease
ncRNA	Non-coding ribonuclease
oGTT	Oral glucose tolerance test
OR	Odds ratio
PTM	Post-translational modifications
RR	Relative Risk

SCT	Syncytiotrophoblast
TFIID	Transcription factor II D
TSS	Transcriptional start site
VDR	Vitamin D receptor
VEGF-A	Vascular endothelial growth factor A
Vit. D	Vitamin D
WB	Western Blot

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5. EIDESTATTLICHE VERSICHERUNG

Paula Sophia Elisabeth, Hepp

Vorname(n), Name

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Titel

„Histologische und experimentelle Untersuchung inflammatorischer und epigenetischer Dysregulation in der Plazenta bei Gestationsdiabetes“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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München, 06.12.2021 — Ort, Datum

Paula Hepp — Unterschrift

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7. PUBLIKATIONSLISTE

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9. ABKÜRZUNGSVERZEICHNIS

BMI	Body Mass Index
CD	Cluster of differentiation
CK7	Cytokeratin 7
CRD	Carbohydrate recognition domain; Kohlenhydrat-erkennende Region
DDG	Deutsche Diabetes Gesellschaft
DGGG	Deutsche Gesellschaft für Gynäkologie und Geburtshilfe
DM2	Diabetes Mellitus Type 2
g	Gramm
GDM	Gestational Diabetes Mellitus
H3K9ac	Acetylierung des 9. Lysins des Histonproteins 3
H3K4me3	Trimethylierung des 4. Lysins des Histonproteins 3
HAT	Histon-Acetyltransferase
HDAC	Histone Deacetylase
HPL	Humanes Plazentalaktogen
IL	Interleukin
IRS	Immunoreactivity score
IUWR	Intrauterine Wachstumsrestriktion
ncRNA	Nicht-codierende RNA
oGTT	Oraler Glukosetoleranztest
PE	Präeklampsie
PP-13	Plazenta Protein 13
TIMP-1	Tissue inhibitor of metallo proteinase 1; Gewebeinhibitor der Metalloprotease 1
TNFα	Tumornekrosefaktor α

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