antioxidants

Special Issue Reprint

The Role of Antioxidants in Pregnant Women's and Children's Health

2nd Edition

Edited by
Magdalena Chełchowska, Jadwiga Ambroszkiewicz and Joanna Gajewska

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Guest Editors

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About the Editors

Magdalena Chełchowska

Magdalena Chełchowska, PhD, DSc (Habilitation), Professor of the Institute of Mother and Child, is Head of the Department of Newborn Screening and Metabolic Diagnostics at the Institute of Mother and Child in Warsaw, Poland. Her scientific and clinical expertise encompasses clinical biology and biochemical diagnostics, with particular emphasis on genetic and metabolic disorders. She leads a team responsible for the implementation and development of Poland's nationwide newborn screening program. She is the author and co-author of approximately 140 peer-reviewed publications and has participated as a principal investigator and collaborator in numerous national and international research projects. Her research interests focus on oxidative stress and antioxidant defense mechanisms, metabolic biomarkers, pregnancy, maternal and child health, maternal tobacco smoking, metabolic diseases, and newborn screening. By integrating clinical observations with biochemical and molecular approaches, her research seeks to elucidate the mechanisms underlying metabolic disturbances and adverse health outcomes during critical periods of human development, as well as to identify reliable biomarkers that may support early diagnosis and preventive strategies. Her work contributes to a better understanding of oxidative and metabolic processes influencing health from the prenatal period through early life.

Jadwiga Ambroszkiewicz

Jadwiga Ambroszkiewicz, PhD, Assistant Professor, is affiliated with the Department of Newborn Screening and Metabolic Diagnostics at the Institute of Mother and Child in Warsaw, Poland. Her research explores the complex interactions between nutritional status, adipose tissue function, bone metabolism, and metabolic health during childhood and adolescence. Her scientific interests include adipokines, myokines, bone metabolism markers, antioxidants, nutrition, child health, and metabolic diseases. She has authored and co-authored 145 publications in peer-reviewed scientific journals and has contributed to research investigating biochemical and metabolic markers associated with nutritional status, lifestyle factors, growth, and development. Her work aims to provide a comprehensive understanding of the biological mechanisms linking nutrition, body composition, tissue function, and metabolic health, with particular interest in the identification of biomarkers of potential diagnostic and preventive value. She is also actively engaged in scientific peer review and serves as a Topical Advisory Panel Member and Reviewer Board Member of Nutrients. Her research contributes to the identification of modifiable nutritional and metabolic factors that may support healthy development and help reduce the risk of metabolic disorders during childhood and adolescence.

Joanna Gajewska

Joanna Gajewska, PhD, Assistant Professor, is affiliated with the Department of Newborn Screening and Metabolic Diagnostics at the Institute of Mother and Child in Warsaw, Poland. She specializes in clinical biochemistry, pediatric metabolic diagnostics, and metabolic diseases affecting children and adolescents. Her research focuses on the biochemical mechanisms underlying metabolic disturbances during childhood, with particular emphasis on the interplay among obesity, adipokines, bone turnover, and oxidative stress. She has authored and co-authored more than 100 peer-reviewed original research articles and review papers addressing the metabolic and biochemical aspects of pediatric health. She has served as both a principal investigator and an investigator in numerous national and international research projects and is an active member of the Polish Society for Obesity Research and the Section of Bone Metabolic Diseases in Children and Adolescents of the Polish Pediatric Society. Her research integrates clinical and biochemical approaches to investigate the relationships among metabolic status, oxidative processes, and biomarkers of tissue function. A particular focus of her work is the identification of biochemical markers that may improve the understanding of metabolic disturbances and support the early recognition, prevention, and management of metabolic disorders in children and adolescents.

Preface

Oxidative stress and disturbances in redox homeostasis are increasingly recognized as important mechanisms underlying a wide range of metabolic, inflammatory, cardiovascular, neurodegenerative, and immune-related disorders. At the same time, growing evidence highlights the importance of antioxidant defense mechanisms, nutrition, and lifestyle factors in maintaining health and preventing disease.

This Reprint brings together recent advances in oxidative stress and antioxidant research, covering molecular mechanisms, redox signaling, biomarkers, nutritional and environmental factors, and emerging therapeutic and preventive approaches. Its scope reflects the interdisciplinary nature of contemporary research, integrating basic, clinical, and translational perspectives.

The Special Issue was motivated by the need to bring together emerging evidence from different fields and to facilitate the exchange of knowledge on the complex interactions between oxidative stress, antioxidant defenses, nutrition, lifestyle, and disease. By highlighting current findings and innovative approaches, the Reprint aims to contribute to a better understanding of redox-related mechanisms and to identify promising directions for future research.

This collection is intended for researchers and clinicians, as well as nutritionists, healthcare professionals, and early-career scientists working in redox biology, biomedical sciences, nutrition, preventive medicine, and related fields. We hope that this Reprint will stimulate further research and interdisciplinary collaboration and contribute to the development of effective strategies for promoting health and preventing oxidative stress-related diseases.

Magdalena Chełchowska, Jadwiga Ambroszkiewicz, and Joanna Gajewska
Guest Editors

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Article

Oxidative Stress Indicated by Nuclear Transcription Factor Nrf2 and Glutathione Status in the Blood of Young Children with Autism Spectrum Disorder: Pilot Study

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Abstract: This pilot study investigated the relationship between nuclear transcription factor Nrf2 and glutathione homeostasis in children with autism spectrum disorder (ASD), addressing the role of oxidative stress in ASD pathophysiology. Oxidative stress, characterized by an imbalance between reactive oxygen species and antioxidant defenses, has been implicated in ASD and may contribute to neuroinflammation and mitochondrial dysfunction. Nrf2, a key regulator of the antioxidant response, influences glutathione synthesis and recycling, making it critical for cellular redox balance. This study included 23 children with ASD and 21 neurotypical healthy controls, and measured levels of Nrf2, Keap1 (Kelch-like ECH-associated protein 1), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), and peroxidase (GPx3) in blood samples. Our study reveals altered antioxidant defense in children with autism spectrum disorder, as evidenced by reduced levels of Nrf2, Keap1, GSH, and GR, along with elevated GSSG and a lower GSH/GSSG ratio. These findings indicate an increased oxidative stress burden in this population. Additionally, the observed positive correlation between Nrf2, GSH, and GR levels suggests an important role for Nrf2 in maintaining glutathione homeostasis. Our results underscore the potential involvement of oxidative stress in ASD and emphasize the need for further research into targeted therapeutic approaches to address this imbalance.

Keywords: autism spectrum disorders; nuclear factor erythroid 2-related factor 2; glutathione; glutathione peroxidase 3; glutathione reductase; oxidative stress; antioxidants

1. Introduction

Autism spectrum disorders (ASDs) are complex neurodevelopmental conditions that manifest in early childhood and are characterized by deficits in communication and social skills as well as repetitive and stereotypical behaviors [1]. There is significant variability among individuals, with each person presenting a unique combination of symptoms and associated health challenges. The multifactorial nature of ASD, involving genetic, environmental, immunological, and neurological factors, makes it difficult to identify a single cause or universal treatment approach. A growing body of evidence highlights the

role of oxidative stress in the pathophysiology of ASD, adding another layer of complexity to our understanding of this disorder [2,3].

Oxidative stress refers to an imbalance in the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the ability of the body to neutralize them with antioxidants. This imbalance can lead to cellular damage that affects various biological systems, including the brain. In people with ASD, oxidative stress is thought to contribute to neuroinflammation, which can exacerbate core symptoms such as communication deficits, social interaction difficulties, and repetitive behaviors [4].

The relationship between oxidative stress, mitochondrial dysfunction, and immune dysregulation is particularly relevant in ASD [5–8]. Mitochondria, the energy producers in cells, are highly susceptible to oxidative damage. Emerging evidence suggests that mitochondrial dysfunction is prevalent in children with ASD, potentially affecting energy metabolism, which is critical for brain development and function. Similarly, immune dysregulation—whether overactive or underactive—can increase inflammation and oxidative stress, leading to the systemic problems often observed in ASD, such as gastrointestinal problems, sleep disturbances, and neurological disorders [9–11].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates the expression of more than 500 genes involved in cellular defense against oxidative stress, particularly those linked to antioxidant pathways. Nrf2 typically binds to Kelch-like ECH-associated protein 1 (Keap1) and remains sequestered in the cytoplasm under non-stress conditions. Keap1 acts as a sensor of oxidative stress through its reactive cysteine residues, which are highly sensitive to ROS and RNS. When oxidative stress levels rise, specific cysteine residues in Keap1 are oxidized, causing a conformational change that releases Nrf2 from the Keap1-Nrf2 complex. Once free, Nrf2 translocates to the nucleus, where it binds to the antioxidant response element (ARE) in the promoter regions of target genes, leading to the transcription of several antioxidant and cytoprotective genes, many of which are involved in glutathione (GSH) metabolism [12,13].

GSH, or γ-glutamylcysteinylglycine, is the primary endogenous non-enzymatic antioxidant and the most abundant thiol in the central nervous system. It plays a vital role in various cellular functions, including transcription, replication, cell growth, enzyme regulation, immune defense, and apoptosis. In the brain, GSH functions as a neurotransmitter, modulates glutamatergic transmission, and protects neurons by chelating metal ions and preventing harmful Fenton reactions. The primary antioxidant function of glutathione involves reducing hydrogen peroxide to water and oxygen and converting organic hydroperoxides into alcohols. During this process, glutathione peroxidase (GPx) oxidizes reduced glutathione (GSH) to glutathione disulfide (GSSG), which is then restored to its reduced form by glutathione reductase (GR). This enzymatic cycle maintains the crucial GSH/GSSG balance for cellular function and survival [6,14–16].

In addition to GSH regeneration from GSSG, glutathione levels are also increased by neosynthesis driven by glutamate-cysteine ligase (GCL). Activated by Nrf2, GCL catalyzes the rate-limiting step in glutathione synthesis by combining glutamate and cysteine, a key sulfur-containing amino acid required for GSH production. Nrf2 further upregulates glutathione S-transferase (GST), an enzyme that uses GSH to detoxify harmful substances, and NADPH oxidoreductase, which maintains the NADPH pool—an essential cofactor for glutathione reductase [17,18].

By upregulating enzymes involved in both the synthesis and recycling of glutathione, Nrf2 plays a critical role in maintaining cellular redox balance. By promoting GSH production and recycling, the Nrf2 pathway enhances the ability of the cell to buffer oxidative stress by neutralizing free radicals and preventing oxidative damage to proteins, lipids, and DNA. This coordinated response between Nrf2 signaling and the GSH/GSSG system en-

sures that cells remain protected from oxidative damage and maintain homeostasis [17,19]. Studies have demonstrated that children with autism have lower levels of reduced glutathione (GSH), higher levels of oxidized glutathione (GSSG), reduced total glutathione (tGSH), and a decreased GSH/GSSG ratio in plasma and brain regions such as the cerebellum and temporal cortex compared to neurotypical children [7,14,20–25]. However, data on Nrf2 levels and Nrf2/Keap1 activity in blood cells from individuals with ASD remain limited and inconsistent in some cases. Findings have revealed both reduced Nrf2 protein expression and activity in the frontal cortex and monocytes, as well as alterations in Nrf2 mRNA levels in peripheral blood mononuclear cells (PBMCs) from individuals with ASD [26–28]. Only one study has compared Nrf2 and Keap1 levels in the serum of children with ASD and healthy controls, focusing on their impact on heme oxygenase-1 (HO-1) expression [29]. In addition, there is a lack of research on the relationship between glutathione status and Nrf2 levels in the blood of children with autism spectrum disorders. The aim of this study was to measure the levels of Nrf2, Keap1 protein, glutathione, and key enzymes involved in its metabolism in the blood of young patients in both autism and neurotypical groups. In addition, this study was an attempt to evaluate the relationship between Nrf2 and markers of glutathione status in both groups.

2. Materials and Methods

2.1. Participants

This study was conducted at the Institute of Mother and Child in Warsaw and included patients from the Clinic of Pediatric Neurology between May 2022 and November 2024. A total of 23 children with ASD (7 girls and 16 boys), aged between 1.6 and 4 years, were included, along with a control group of 21 neurotypical healthy children (8 girls and 13 boys) of the same age. All participants were Caucasian. The diagnosis of ASD was confirmed based on the 10th revision of the International Classification of Diseases (ICD-10) by pediatric psychiatrists, supported by psychological examination and observation [30]. Children with ASD underwent neurological observation and differential diagnosis in the pediatric neurology unit to avoid misdiagnosis.

The inclusion criteria for the study group were a confirmed diagnosis of ASD, normal neurological findings, and a history of uncomplicated pregnancy and delivery. Exclusion criteria included any chronic illness requiring long-term treatment; significant hearing or visual impairment; current antibiotic therapy; or the use of anti-inflammatory, anti-allergy, antioxidant, or immunomodulatory drugs/supplements. Additional exclusion criteria were based on the presence of an elimination diet or acute or chronic infection within one month prior to blood collection. The control group had the same inclusion and exclusion criteria, except for the diagnosis of autism spectrum disorder.

This study was carried out in accordance with the ethical standards of the Declaration of Helsinki after approval by the Ethics Committee of the Institute of Mother and Child (decision number 16/2022, date of approval 5 May 2022). Parents of all participants were informed of the aims and procedures of this study and provided written consent for the analysis of biological samples and use of clinical information from the patients' medical records.

2.2. Blood Processing

For biochemical assessments, 4 mL of venous blood was collected into EDTA-containing vacutainer tubes from each participant early in the morning after an overnight fast. Blood samples were processed according to the specified requirements for biochemical analysis. After centrifugation, the resulting plasma was divided into small portions and immediately stored at −80 °C for future analysis.

2.3. Biochemical Analysis

Plasma levels of antioxidant defense markers were measured using precise immunoenzymatic assays, strictly following the manufacturer's guidelines at every step.

The concentrations of nuclear factor erythroid-derived 2-like 2 (NFE2L2 or Nrf2) and Keap1 were measured using ELISA kits (Human NFE2L2 ELISA Kit Cat. No.: EH3417 and Human KEAP1 ELISA Kit Cat. No.: EH4240, Fine Biotech Co., Ltd., Wuhan, China) based on sandwich ELISA technology. Both assays followed a similar procedure: Monoclonal antibodies specific to NFE2L2 or Keap1 were pre-coated onto 96-well plates. Samples and standards were added to the wells, and after incubation, unbound substances were washed away. Biotinylated detection antibodies specific to NFE2L2 or Keap1 were then added. After removing the excess detection antibodies, horseradish peroxide Streptavidin conjugates were added. The colorimetric reaction was initiated by adding TMB (3,3′,5,5′-tetramethylbenzidine), which reacted with HRP to produce a blue color. The reaction was stopped by adding a stop solution, turning the color from blue to yellow. The intensity of the yellow color, measured at 450 nm using a microplate reader, was directly proportional to the concentration of the target protein in the samples. The concentrations of NFE2L2 and Keap1 were calculated by comparing the optical density (OD) values at 450 nm with a standard curve. The intra- and inter-assay coefficients of variation for NFE2L2 and Keap1 were less than 8.0% and 10%, respectively, with detection sensitivities below 0.094 ng/mL for NFE2L2 and 14.063 pg/mL for Keap1.

GSH and GSSG levels were quantified using sandwich enzyme-linked immunosorbent assay (ELISA) kits using two highly specific monoclonal antibodies (Human GSH ELISA Kit, Cat. No.: 201-12-5407; Human GSSG ELISA Kit, Cat. No. 201-12-5444, SunRed Bio-Technology Company, Shanghai, China). The cellular redox index was assessed by calculating the GSH/GSSG ratio (R).

The levels of glutathione reductase (GR) and glutathione peroxidase 3 (GPx3) were similarly determined using appropriate ELISA kits (Human Glutathione Reductase ELISA Kit, Cat. No.: MBS2703164, and Human Glutathione Peroxidase 3 ELISA Kit, Cat. No: MBS2881280, MyBioSource Inc., San Diego, CA, USA). The intra- and inter-assay coefficients of variation were less than 8.0% and 10% for GPx3 and 10% and 12% for GSH, GSSG, and GR. The detection sensitivities of the assays were less than 0.118 μmol/L for GSH, 0.045 μmol/L for GSSG, 0.230 ng/mL for GPx3, and 35.000 pg/mL for GR. Detailed descriptions of these methods can be found in our previous studies [31].

2.4. Statistical Analysis

Baseline characteristics were presented as means with standard deviation or medians with interquartile range, depending on whether the data were normally distributed. The correlation between the studied variables was assessed using Spearman's coefficient. The distributions of the values of the analyzed parameters in the autistic children and control groups were compared using the Mann–Whitney (MW) test. The level of difference between groups was assessed by the r effect size measure, calculated as the standardized MW test (z) divided by the root of N, with 0.30, 0.50, and 0.80 interpreted as small, medium, and large effect sizes, respectively. In addition, a general linear model (GLM) was used, examining the main effect of age, sex, and group membership and the 2 -way interaction between gender or age and group as predictors of each parameter. Statistical Package for the Social Sciences (SPSS) Version 29.0 (IBM Corp, Armonk, NY, USA) was used, and p values <0.05 were considered statistically significant.

3. Results

The mean age of the children in the two study groups did not differ significantly ($p = 0.671$). The average age in the autism group was 3.2 years, while in the control group it was 2.9 years. There were also no sex differences between the groups ($p = 0.592$).

The differences in the plasma concentrations of the antioxidant parameters between the two groups are presented in Table 1.

Table 1. Comparison of antioxidant defense markers between children with autism and healthy controls.

	Control Children $n = 21$	**Autistic Children** $n = 23$	***p*-Value**
[a] GSH (μmol/L)	16.44 ± 0.91	14.78 ± 2.80	0.037
[a] GSSG (μmol/L)	3.19 ± 0.35	4.52 ± 1.46	0.001
[b] R (GSH/GSSG)	5.17 [4.68–5.59]	3.53 [2.68–4.53]	0.000
[a] GPx3 (ng/mL)	18.00 ± 2.28	17.47 ± 4.62	0.366
[b] GR (pg/mL)	300.52 [282.83–309.45]	280.11 [228.73–301.23]	0.019
[a] Nrf2 (ng/mL)	1.98 ± 0.22	1.72 ± 0.39	0.013
[a] Keap1(pg/mL)	171.48 ± 25.09	149.08 ± 31.98	0.005

[a] mean values and standard deviation (SD); [b] median values and interquartile range (1-3IQR); GSH—reduced glutathione; GSSG—oxidized glutathione; GPx3—glutathione peroxidase; GR—glutathione reductase, R (GSH/GSSG ratio)—index of the cell's redox; Nrf2—nuclear factor erythroid-derived 2-like protein 2; Keap1—Kelch-like ECH-associated protein A.

Children with autism had significantly lower plasma levels of GSH, GR, Nrf2, and Keap1 ($p < 0.05$), as well as a lower R-index value ($p < 0.001$), compared to the healthy children. Conversely, GSSG concentrations were significantly higher in the autism group ($p < 0.01$), whereas GPx3 levels were not significantly different between the groups.

Figure 1 shows the differences between the two groups as expressed by the r effect size measure, except GPx3, for which no statistically significant difference was found. The largest difference was shown for R and GSSG, where the r value exceeded 0.5, indicating a medium effect. For the other four parameters, the differences were significant, but the effect size qualified them at a small level.

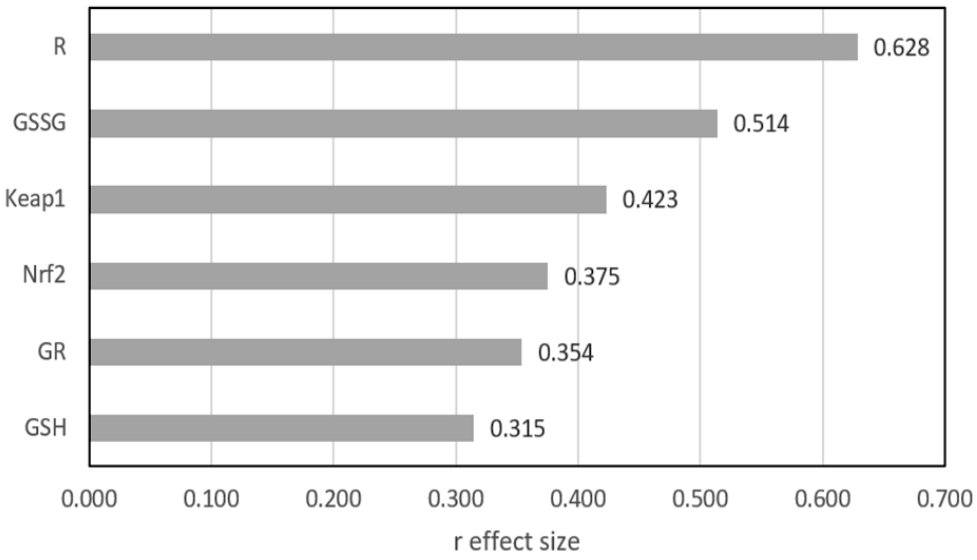


Figure 1. Mann–Whitney r effect size comparison of two groups. GSH—reduced glutathione; GSSG—oxidized glutathione; GPx3—glutathione peroxidase; GR—glutathione reductase, R (GSH/GSSG ratio)—index of the cell's redox; Nrf2—nuclear factor erythroid-derived 2-like protein 2; Keap1—Kelch-like ECH-associated protein A.

It was confirmed that neither sex nor age had an impact on the inference of differences between the groups. The results of the GLM estimation indicate a statistically significant difference between the study and control groups for all parameters except GPx3. None of the seven models showed a significant main effect of sex or age on the variability of the respective parameter, nor a significant two-way interaction between sex or age and group.

Table 2 presents a detailed analysis of the correlations between selected antioxidant parameters for the entire group of children, as well as for each group separately. In the whole group, plasma GSH concentrations positively correlated with GR, R-index, Nrf2, and Keap1 levels. Similarly, Nrf2 levels were significantly associated with Keap1, GR, and R-index. A positive correlation was also found between GR and GPx3 levels. Conversely, GSSG levels were negatively correlated with the R-index.

Table 2. Spearman's rank correlation coefficients between antioxidant defense markers in the entire group of studied children (n = 44), the control group (n = 21), and the ASD group (n = 23).

	Entire Group n **= 44**		**Control Group** n **= 21**		**ASD Group** n **= 23**	
	rho	***p*-Value**	***rho***	***p*-Value**	***rho***	***p*-Value**
GSH/GSSG	−0.014	0.929	0.158	0.493	0.150	0.494
GSH/R	0.429	0.004	0.340	0.132	0.333	0.121
GSH/GR	0.632	0.000	0.613	0.003	0.591	0.003
GSH/GPx3	0.212	0.167	−0.377	0.092	−0.022	0.082
GSH/Nrf2	0.493	0.001	0.401	0.071	0.394	0.078
GSH/Keap1	0.453	0.002	0.377	0.096	0.277	0.200
GSSG/R	−0.855	0.000	−0.841	0.000	−0.814	0.000
GSSG/GR	−0.252	0.099	−0.368	0.101	−0.083	0.707
GSSG/GPx3	−0.095	0.540	0.109	0.638	−0.022	0.922
GSSG/Nrf2	−0.052	0.391	−0.052	0.823	0.024	0.914
GSSG/Keap1	−0.133	0.457	0.016	0.944	0.186	0.396
R/GR	0.512	0.000	0.593	0.005	0.329	0.125
R/Gpx3	0.162	0.292	−0.094	0.685	0.210	0.612
R/Nrf2	0.358	0.017	0.259	0.258	0.112	0.335
R/Keap1	0.253	0.097	0.199	0.388	−0.087	0.691
GR/GPx3	0.345	0.022	0.049	0.832	0.571	0.004
GR/Nrf2	0.530	0.000	0.465	0.034	0.430	0.041
GR/Keap1	0.305	0.044	0.432	0.050	0.029	0.897
Gpx3/Nrf2	0.071	0.071	0.060	0.797	0.009	0.968
Gpx3/Keap1	0.180	0.180	0.045	0.845	0.219	0.315
Nrf2/Keap1	0.408	0.006	0.319	0.066	0.175	0.733

GSH—reduced glutathione; GSSG—oxidized glutathione; GPx3—glutathione peroxidase; GR—glutathione reductase, R (GSH/GSSG ratio)—index of the cell's redox; Nrf2—nuclear factor erythroid-derived 2-like protein 2; Keap1—Kelch-like ECH-associated protein A.

Figures 2–4 illustrate the significant associations between the studied parameters in both the control and ASD groups. In both groups, GR levels demonstrated a positive correlation with GSH (Figure 2) and Nrf2 concentrations (Figure 3). Moreover, a negative correlation was observed between GSSG levels and the R-index (Figure 4).

Additionally, in the ASD group, a positive correlation was observed between GR and GPx3 levels ($p < 0.01$). Meanwhile, in the control group, GR levels were positively correlated with R-index values ($p < 0.01$). No other correlations were confirmed between antioxidant defense parameters in the two groups.

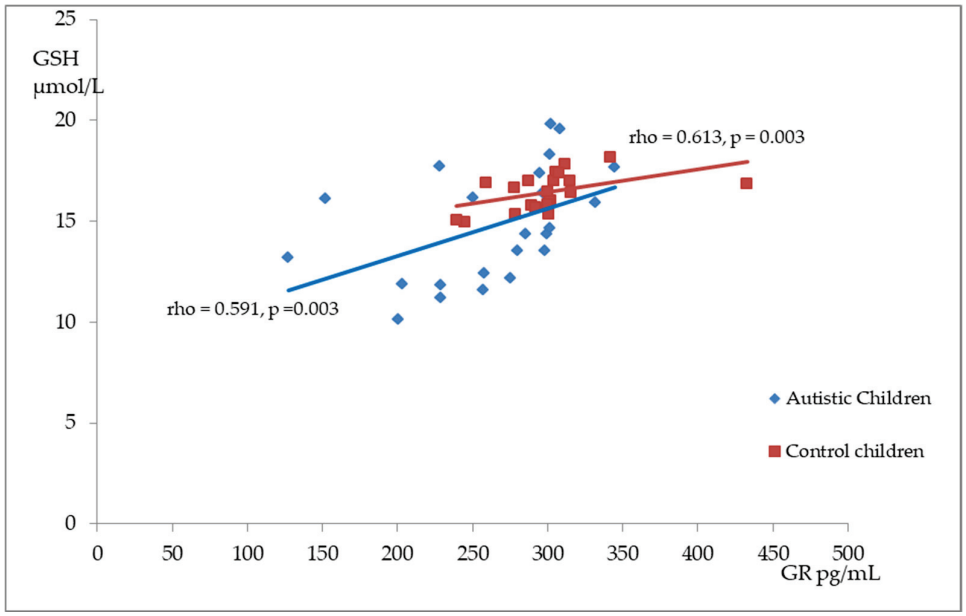


Figure 2. A scatterplot illustrating the correlation between reduced glutathione (GSH) and glutathione reductase (GR) levels in children with autism compared to the control group.

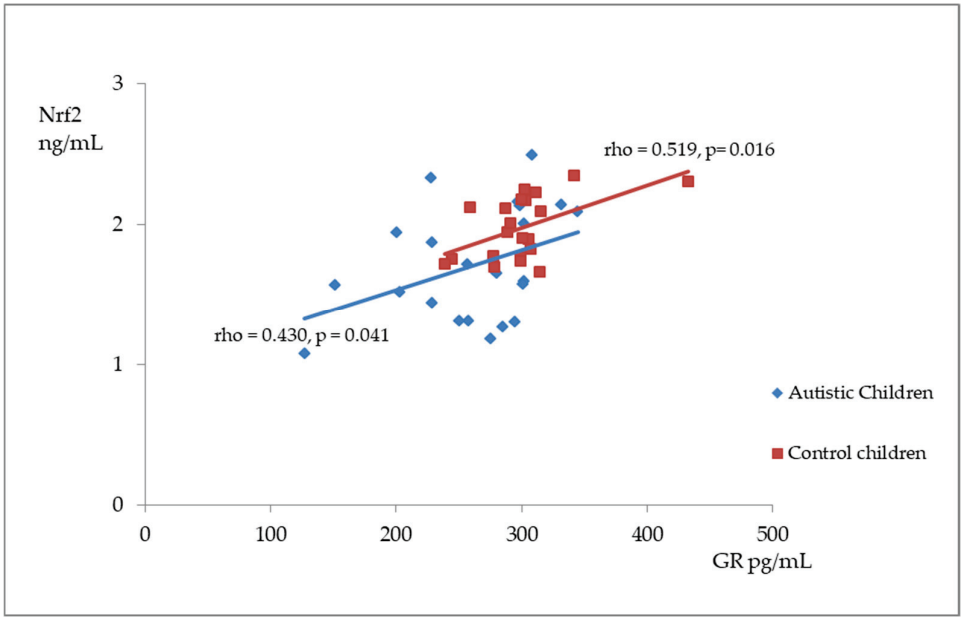


Figure 3. A scatterplot illustrating the correlation between nuclear factor erythroid-derived 2-like 2 (Nrf2) and glutathione reductase (GR) levels in children with autism compared to the control group.

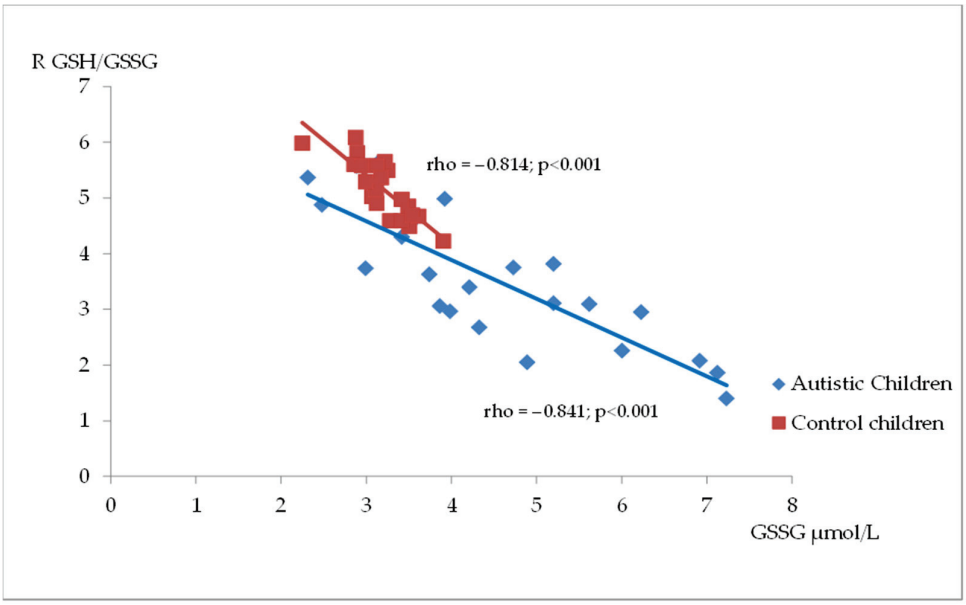


Figure 4. A scatterplot illustrating the correlation between oxidized glutathione (GSSG) and GSH/GSSG ratio (R) levels in children with autism compared to the control group.

4. Discussion

Oxidative stress in ASD is characterized by reduced activity of antioxidant enzymes (superoxide dismutase, SOD, catalase, CAT, and GPx) and increased markers of cellular damage, such as DNA oxidation (8-hydroxy-2′-deoxyguanosine, 8OHdG), lipid peroxidation (malondialdehyde, MDA, and thiobarbituric acid-reactive substances, TBARSs), and protein oxidation (carbonyl proteins) [2,32–36]. Important biomarkers of oxidative stress in ASD are advanced glycation end products (AGEs). The observed accumulation of plasma protein glycation and oxidation markers in ASD may be significant in predicting disease progression [37,38].

The results of our study indicated reduced Nrf2 levels, accompanied by diminished reduced glutathione and an altered GSH/GSSG ratio, confirming the presence and severity of oxidative stress in young children with autism. Additionally, the observed positive correlation between Nrf2 levels and GSSG and GR concentrations in the entire study group suggests significant relationships between these parameters.

Reduced Nrf2 expression has been linked to several brain disorders, including Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease, and its function appears to be weakened in depression [39–42]. Evidence suggests that Nrf2 levels and Nrf2/Keap1 activity may also be disrupted in autism spectrum disorders [30,31]. In our study, we found significantly lower levels of Nrf2 and Keap1 proteins in the plasma of autistic children than in neurotypical healthy children. These results are consistent with an in vitro study by Nadeem et al. [26,43], who examined blood cells from autistic children. Their findings showed that children with ASD have reduced Nrf2 protein expression and nuclear binding activity in monocytes and neutrophils compared with typically developing children. This decrease could be associated with increased inflammation and nitrative stress, potentially characterized by higher levels of pro-inflammatory cytokines (IL-6 and IL-1β) and impaired antioxidant responses to environmental stressors, which may contribute to the oxidative imbalance observed in ASD [26,43]. On the other hand, a more recent study by Bolotta et al. [28] found a modest but significant increase in Nrf2 mRNA levels in PBMCs from individuals with ASD. This raises the possibility that, despite elevated mRNA levels, there may be a defect in Nrf2′s ability to translocate to the nucleus. Nrf2 expression and activity are also downregulated in the frontal cortex, a region that

plays a crucial role in higher cognitive functions such as decision-making, problem-solving, planning, attention, and the regulation of emotions and behavior [27]. In contrast, one study found elevated serum levels of Keap1 and Nrf2 in children with autism along with reduced levels of heme oxygenase-1 (HO-1), a key antioxidant enzyme whose gene expression is regulated by Nrf2 [29]. These discrepancies may result from the complex regulation of the Nrf2/Keap1 pathway in response to oxidative stress. Reduced Nrf2 and Keap1 activity or levels in autistic children may indicate protein depletion after excessive oxidative stress, while elevated levels could reflect an earlier phase of the response, where these proteins are upregulated to counteract oxidative damage.

As previously mentioned, malfunction of the Nrf2/Keap1 pathway, which controls Nrf2 stability and activation in response to oxidative stress, may further contribute to impaired antioxidant defenses, including the maintenance of adequate glutathione levels [17,18].

The mitochondrial abnormalities and inflammation found in autism are sources of elevated hydrogen peroxide levels, potentially leading to GSH dysregulation. Similar to other authors, we found lower levels of reduced glutathione and the GSH/GSSG redox index and elevated concentrations of oxidized glutathione in the plasma of children with ASD compared with healthy children [22,32,44,45]. The effect size measure used in our analyses, which ranks parameters according to the magnitude of differences between groups, proved particularly useful given the small sample size. It demonstrated that the effect size for the R-index and oxidized glutathione was notably larger than for the other parameters. Additionally, similar to others, we observed a strong negative association between these two parameters, indicating intracellular oxidative stress in children with ASD [22].

To the best of our knowledge, the positive correlation we observed for the first time between Nrf2 levels and both GSH concentrations and the GSH/GSSG ratio in the plasma of the studied children may confirm the role of this factor in maintaining glutathione homeostasis.

In contrast, we found no differences in GPx levels between the groups, likely because we measured the extracellular form of glutathione peroxidase (GPx3), which is primarily found in plasma, while others have assessed total GPx activity [32,36,46]. However, we demonstrated reduced GR activity in individuals with ASD, which contributed to the observed imbalance in the GSH/GSSG ratio in this group. As GR levels declined, we observed a corresponding reduction in reduced glutathione. Additionally, there was a positive correlation between GR and Nrf2 levels in both groups. The lower GR levels in the ASD group may result not only from reduced GR gene expression but also from a decreased expression of genes involved in nicotinamide adenine dinucleotide phosphate (NADPH) production and regeneration. Nrf2 regulates key enzymes of the pentose phosphate pathway, such as G6PD (glucose-6-phosphate dehydrogenase) and PGD (phosphogluconate dehydrogenase), which are the primary sources of NADPH in cells. By promoting NADPH production, Nrf2 supports antioxidant defenses and facilitates GSH regeneration from GSSG, enabling cells to manage oxidative stress better [13].

The decrease in GSH levels can also result from impaired de novo synthesis within the cell, which depends on the availability of key amino acids, such as cysteine and methionine, both of which are critical for glutathione production [47,48]. Cysteine plays a direct role in GSH synthesis, whereas methionine, through its involvement in the methionine cycle, can be converted into cysteine via the transsulfuration pathway. A recent meta-analysis reported significantly lower plasma concentrations of methionine and cysteine in individuals with ASD than in healthy controls [3]. Although we lacked specific data on the plasma levels of these amino acids in the children included in our study, this represents a limitation. However, the reduced level of plasma Nrf2 and Keap1 protein observed

in our study may contribute to the altered expression of glutamate-cysteine ligase, the rate-limiting enzyme in de novo glutathione synthesis, further impacting GSH levels and cellular redox balance.

In humans, aging is associated with a decline in antioxidant defenses, as evidenced by reduced total GSH levels, lower GSH/GSSG ratios, and diminished activity of enzymes like GPx and GR, which contribute to increased oxidative stress and may play a role in the development of neurodegenerative diseases [49,50]. Studies have also shown a negative correlation between Nrf2 levels and age in children with ASD, particularly in those aged 3 to 12 years [29]. However, in our study, we did not observe an age-related impact on any of the examined parameters. This could be due to the fact that our research focused on a group of very young children, all within a narrow age range, which may limit the detection of age-related differences.

Also, sex differences in GSH metabolism have been observed in both animal models and humans [49,51–53]. In rats, females exhibited higher levels of hepatic mitochondrial GSH and increased GPx activity compared to males, but these levels decreased following ovariectomy [51]. However, our study did not find a significant sex-based effect on oxidative stress markers in young children with ASD, which contrasts with others studies suggesting that male mitochondria are more vulnerable to oxidative stress due to lower levels of reduced glutathione [52].

A limitation of our study is the small sample size, which may have reduced the significance of our findings. However, we conducted this study on a group of very young Caucasian children (average age: 3 years, range: 1.6–4.0 years) who had just been diagnosed with autism. As a result, these children had not undergone prior treatments, including antioxidant, anti-allergic, or immunomodulatory therapies. Since some oxidative stress parameters can be influenced by age and sex, the study and control groups were matched to avoid potential bias [49–52]. Despite the small sample size, this study serves as a valuable starting point for investigating oxidative stress within the broader context of metabolic research. This includes profiling amino acids, purines, pyrimidines, and organic acids, as well as considering allergological, genetic, and immunological factors. Our findings will help guide future research, providing insights into the interactions between oxidative stress and other metabolic disruptions in a larger group of young children with ASD.

5. Conclusions

In conclusion, our study provides evidence of impaired Nrf2 and glutathione homeostasis in children with autism spectrum disorder, highlighting a potential role of oxidative stress and ASD. Low Nrf2 levels, along with reduced glutathione and an altered GSH/GSSG ratio, may reflect an insufficient antioxidant response to oxidative stress in ASD. Furthermore, the decreased glutathione reductase activity and the significant correlation between Nrf2 and GR suggest that Nrf2 may regulate GR expression, contributing to the redox imbalance observed in children with autism spectrum disorder. These findings establish a basis for future research into the neuroimmunometabolic aspects of ASD and may offer valuable insights into potential therapeutic strategies.

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antioxidants

Article

Therapeutic Effect of Alpha Lipoic Acid in a Rat Preclinical Model of Preeclampsia: Focus on Maternal Signs, Fetal Growth and Placental Function

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Abstract: Chronic hypertension is a major risk factor for preeclampsia (PE), associated with significant maternal and neonatal morbidity. We previously demonstrated that pregnant stroke-prone spontaneously hypertensive rats (SHRSP) display a spontaneous PE-like phenotype with distinct placental, fetal, and maternal features. Here, we hypothesized that supplementation with alpha lipoic acid (ALA), a potent antioxidant, during early pregnancy could ameliorate the PE phenotype in this model. To test this hypothesis, timed pregnancies were established using 10 to 12-week-old SHRSP females (n = 19–16/group), which were assigned to two treatment groups: ALA (injected intraperitoneally with 25 mg/kg body weight ALA on gestation day (GD1, GD8, and GD12) or control, receiving saline following the same protocol. Our analysis of maternal signs showed that ALA prevented the pregnancy-dependent maternal blood pressure rise (GD14 blood pressure control 169.3 ± 19.4 mmHg vs. 146.1 ± 13.4 mmHg, p = 0.0001) and ameliorated renal function, as noted by the increased creatinine clearance and improved glomerular histology in treated dams. Treatment also improved the fetal growth restriction (FGR) phenotype, leading to increased fetal weights (ALA 2.19 ± 0.5 g vs. control 1.98 ± 0.3 g, p = 0.0074) and decreased cephalization indexes, indicating a more symmetric fetal growth pattern. This was associated with improved placental efficiency, decreased oxidative stress marker expression on GD14, and serum soluble fms-like tyrosine kinase 1 (sFlt1) levels on GD20. In conclusion, ALA supplementation mitigated maternal signs and improved placental function and fetal growth in SHRSP pregnancies, emerging as a promising therapy in pregnancies at high risk for PE.

Keywords: preeclampsia; placental dysfunction; animal model; antioxidant therapy; endothelial dysfunction; inflammation

1. Introduction

Preeclampsia (PE) is a pregnancy-specific disorder that affects 2 to 8% of pregnancies worldwide and represents a leading cause of maternal and perinatal mortality [1]. PE usually manifests after the 20th week of pregnancy with hypertension, signs of maternal end-organ damage (i.e., kidney or liver dysfunction), and fetal growth restriction (FGR) [2]. There is growing awareness regarding the long-term consequences of PE, as it is associated with an increased risk of cardiometabolic disorders, renal disease, and other chronic conditions both in mothers and their offspring [3].

Chronic hypertension is a major risk factor for PE [4], and adverse maternal and perinatal outcomes are more prevalent when PE is superimposed on pre-existing hypertension than for de novo disease [5]. Management of this high-risk group is still challenging, as many women with chronic hypertension are recommended to modify their medication upon conception due to the potential teratogenic effects of some antihypertensive therapies [6]. Current treatment options include antihypertensive therapy to avoid progression to severe hypertension and low-dose aspirin for prevention of superimposed PE, but many women are non-responsive [7]. Furthermore, recent animal studies showed that standard antihypertensive therapies used in PE patients fail to improve maternal hemodynamics and cardiac compliance and, therefore, reduce maternal cardiovascular risk [8]. Therefore, the development of safe treatment alternatives that can help minimize the risk of adverse outcomes in hypertensive pregnancies is an acute need.

While the origin of PE is still unclear, the placenta seems to play a central pathogenic role. Currently, PE is considered a multifactorial syndrome involving placental malperfusion with ensuing oxidative stress, an exaggerated inflammatory response, and angiogenic factor imbalance [9]. The release of antiangiogenic soluble fms-like tyrosine kinase 1 (sFlt1) by the ischemic placenta causes generalized endothelial dysfunction, intravascular inflammation, and activation of the coagulation system [10]. Targeting these mechanisms may be the key to developing an effective pharmacological treatment for pregnancies affected by PE. In particular, antioxidant treatment (i.e., with vitamins E/C, melatonin, or other supplements) has long been proposed as an option for PE. However, the results of the many trials conducted so far have been disappointing [11]. Over 15 trials in the past two decades have evaluated the use of antioxidant supplements in hypertensive disorders of pregnancy (as reviewed in [11]) but showed no conclusive evidence of their beneficial effect. In this context, the 'master antioxidant' alpha lipoic acid (ALA) has been hypothesized as a potential candidate for the treatment of PE due to its diversity of effects, including among others the ability to scavenge free radicals and regenerate endogenous antioxidants [12], promote the expression of antioxidant enzymes [13], mitigate endoplasmic reticulum stress [14], inhibit inflammatory signaling cascades [15,16] and enhance nitric oxide availability [17]. However, to date, this hypothesis lacks formal testing in preclinical or clinical settings.

Preclinical animal models represent an essential prerequisite for the validation of novel therapeutic interventions. Our previous studies have described the stroke-prone spontaneously hypertensive rat (SHRSP) as a preclinical model of PE with distinct placental, maternal, and fetal features [18–20]. Pregnant SHRSP spontaneously display increased blood pressure, signs of renal compromise including decreased urine output, and eventual proteinuria and asymmetric FGR [18]. These features develop secondarily to impaired placentation with defective trophoblast invasion [18], increased production of sFlt1, and activation of placental oxidative stress pathways [19,20]. Considering these findings, we hypothesized that ALA supplementation during the placentation period would ameliorate PE-like features in pregnant SHRSP [21]. To test this hypothesis, we evaluated the effects of ALA treatment on maternal blood pressure and renal function parameters, fetal outcomes, placental morphology, and placental levels of oxidative stress, inflammation, and angiogenic markers.

2. Materials and Methods

An expanded Materials and Methods section is available as Supplemental Material.

2.1. Animals and Experimental Design

Timed pregnancies were established using SHRSP females (10–12 weeks old, 200–250 g body weight n = 19–16 animals/group) as described previously [18]. Matings were established so as to achieve an n = 8–10 animals/treatment group for each time point analyzed. The number of animals was based on previously published studies using the same model [18,19]. Females were randomly paired to littermate males (at a 1:1 ratio) using a computer-based random order generator. Upon vaginal plug detection (GD1), dams were assigned to two groups: ALA treatment (injected intraperitoneally with 25 mg/kg ALA on GD1, GD8, and GD12) and controls, receiving saline under the same protocol. Allocation to the treatment groups was performed using a minimization strategy so that there was a balanced distribution of animals to the treatment groups throughout the study. The timing of ALA administration was set empirically to encompass the placentation period (which is completed by GD12) while minimizing animal distress during the peri-implantation period due to manipulation and injection. The treatment dose was set in agreement with previous reports on the administration of ALA to pregnant rats [22]. Maternal blood pressure profiles throughout pregnancy were recorded using a noninvasive volume pressure recording device, as reported previously [18]. Briefly, blood pressure records were taken at baseline (pre-mating), GD1, GD7, GD14 (onset of disease), and GD18 (late gestation, fully established maternal syndrome). Records were made in the morning, with five cuff inflations as acclimation followed by 20 measurement cycles. Animals were euthanized on GD14 or GD20 for collection of fetoplacental specimens and kidneys used for morphological and molecular analyses. Maternal weight and the specific weight gain (normalized to litter size) during pregnancy were determined on GD20. Further details of the experimental protocol, including raw data for blood pressure profiles and fetal growth parameters, are available in the Supplemental Material (Tables S2–S4).

2.2. Sex as a Biological Variable

Maternal parameters (BP, weight gain, renal function, and serum biomarkers) were determined in females because the disease modeled is only relevant in females. For fetal and placental determinations, sex was not considered as a biological variable.

2.3. Fetal Phenotype Evaluation

Fetal development was assessed morphometrically on GD20. Briefly, following the collection of amniotic fluid, the amnion was dissected to expose the fetuses and placentas, which were fixed in phosphate-buffered 10% formaldehyde (pH 7.2). Placental and fetal weights were recorded from the fixed specimens, and fetal head diameter was measured using a Vernier caliper. The cephalization index (head circumference to body weight ratio) was calculated as an indicator of asymmetric fetal growth, as described in our previous publication [18].

2.4. Histology and Immunohistochemistry

Morphometrical evaluation of placental and kidney samples was performed as described in our previous publication [18], using QuPath v0.4.4 (https://qupath.github.io/, accessed on 23 April 2024) [23] and Angiotool (https://ccrod.cancer.gov/confluence/display/ROB2/Home, accessed on 23 April 2024) [24] for quantitative image analysis.

2.5. Western Blots

Expression of NLRP3, SOD2, and NOX 1 and 2 subunits (NOX1, NOXO1, p47phox, gp91phox) was evaluated in placental extracts following a previously published western blot protocol [19]. Images of the full unedited blots are included in the Supplemental Material.

2.6. Placental Gene Expression Analysis

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate the mRNA levels of caspase 1 (*Casp1*), interleukin (IL) 1β (*Il1b*), IL18 (*Il18*),

Flt1 (*Flt1*), vascular endothelial growth factor (*Vegfa*), and hypoxia-inducible factor 1 alpha (*Hif1a*) as published [25]. Detailed protocols and primer sequences are included in the Supplemental Material (Table S1).

2.7. Statistical Analysis

Results are expressed as mean ±SD or median (minimum–maximum), with n representing the number of individual experiments, pregnant dams, or fetuses. Outliers were identified using the ROUT method (Q = 5%) [26], which allows simultaneous detection of multiple outliers based on robust nonlinear regression and the false discovery rate. Data were checked for normality using the Shapiro–Wilk test. Student's *t*-test or Mann–Whitney U-tests were used to analyze normally distributed and skewed data, respectively. Graphing and calculations were performed using the GraphPad Prism 8.0 software (https://www.graphpad.com/, accessed on 23 April 2024). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Treatment with ALA Prevents Maternal Blood Pressure Elevation and Mitigates Renal Damage in SHRSP Pregnancies

To investigate its therapeutic potential in PE, we designed a protocol for ALA supplementation in the SHRSP model (Figure 1A) and evaluated the effects of treatment on maternal signs, fetal growth, and placental function. Compared to the control treatment, ALA prevented the pregnancy-dependent maternal blood pressure elevation in the SHRSP model, as shown by the significantly decreased systolic blood pressure observed on GD14 (169.3 ± 4.3 mmHg vs. 146.1 ± 3.4 mmHg, $p = 0.0001$, Figure 1B and Figure S1A). Treated dams also showed a significant increase in the creatinine clearance (Cr Cl) on GD20 (control 1.36 ± 0.17 mL/min vs. ALA 1.62 ± 0.15 mL/min, $p = 0.037$, Figure 1C), but no differences were observed in the albumin-to-creatinine ratio (ACR, 0.51 ± 0.07 vs. 0.54 ± 0.08, $p = 0.4705$) or urine output compared to controls (Figure 1C and Figure S1B). Histological evaluation of the kidneys on GD20 revealed a marked decrease in mesangial extracellular matrix accumulation in treated dams (median PAS-positive area control 53.81% vs. ALA 30.29%, $p < 0.0001$, Figure 1D, left panel), but no significant differences in the glomerular expression of podocin ($p = 0.1213$, Figure 1D, right panels). The treatment produced no significant effect on maternal weight or the specific weight gain during pregnancy ($p = 0.8598$ and $p = 0.4412$, respectively, Figure S1C).

3.2. ALA Supplementation Rescues the Asymmetric FGR Phenotype in the SHRSP Model

Since SHRSP pregnancies are characterized by asymmetric FGR [18,27], we next assessed biometric parameters on GD20 to determine the effect of ALA on the fetal phenotype. Figure 2A shows representative examples of fetuses collected from ALA-treated dams (left panel), which displayed significantly increased body weights compared to the controls (2.19 ± 0.28 g vs. 1.98 ± 0.48 g, $p = 0.0074$, Figure 2A). Furthermore, in an analysis performed to verify the reproducibility of our previous observations [18], we observed that ALA treatment was able to restore fetal weight in the SHRSP model, showing no significant differences compared to the normotensive control strain (Figure S2A). The cephalization index, which, when increased, indicates a 'brain-sparing' effect consistent with asymmetric FGR [27], was significantly decreased upon ALA treatment (control 1.63 ± 0.19 cm/g vs. ALA 1.53 ± 0.24 cm/g, $p = 0.0181$, Figure 2B and Figure S2B). The improved fetal phenotypes observed in ALA-treated animals were associated with a significant increase in placental efficiency (measured as the fetal to placental weight ratio, control 4.92 ± 0.13 vs. ALA 5.49 ± 1.17, $p = 0.0453$, Figure 2C) and placental weight (control 0.39 ± 0.05 g vs. ALA 0.41 ± 0.05 g, $p = 0.0236$, Figure S2C).

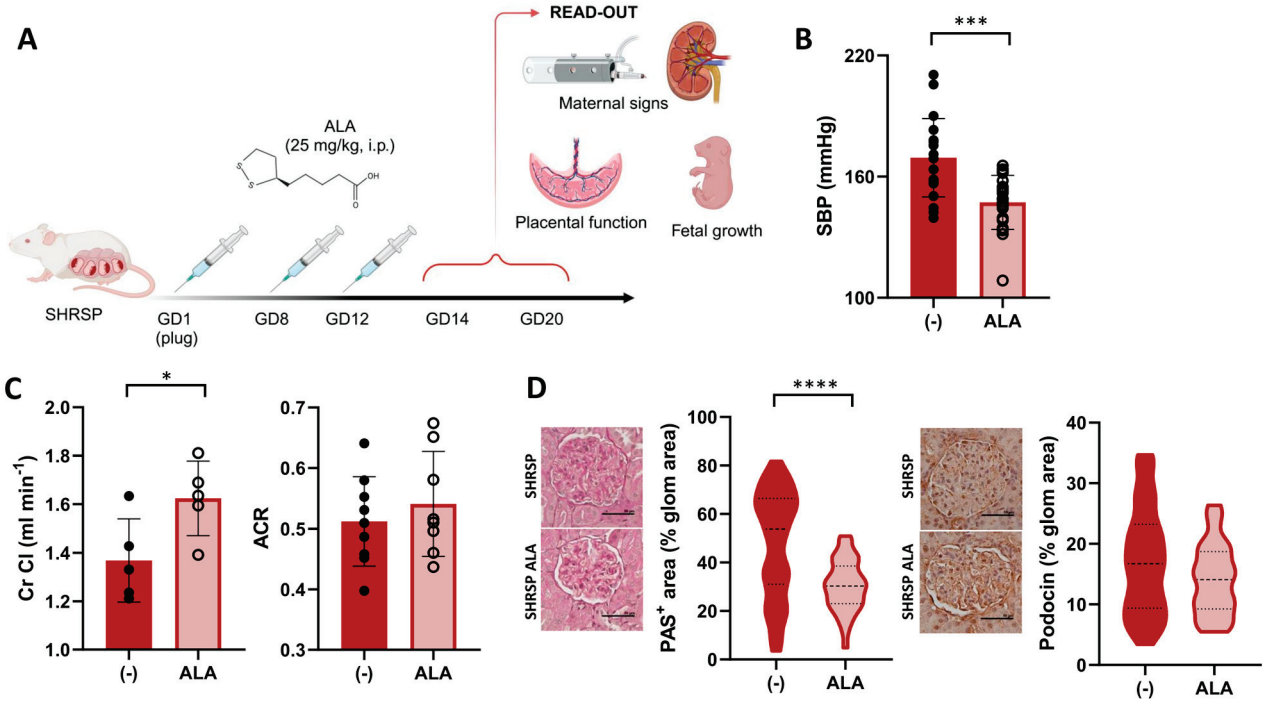


Figure 1. ALA improves maternal PE signs in SHRSP pregnancies. (**A**) Scheme of the experimental protocol for ALA supplementation. Panel created with Biorender (**B**) Maternal systolic BP (SBP) in ALA-treated SHRSP, measured on GD14. Data are individual measurements and mean ± SD, n = 20–21/group, *** $p < 0.001$ analyzed by t-test. (**C**) Creatinine clearance (Cr Cl, left graph) and urine albumin-to-creatinine ratio (ACR, right graph) assessed on GD20. Data are individual measurements and mean ± SD, n = 5–9/group, * $p < 0.05$ analyzed by t-test. (**D**) Histological assessment of renal damage in ALA-treated dams. Mesangial matrix expansion (PAS-positive stain, left graph) was decreased upon ALA treatment. Glomerular expression of podocin (right graph), assessed by immunohistochemistry, was not modified by the treatment. Scale bar: 50 µm. Data are medians (dashed line) and minimum–maximum, n = 5–6 samples/group analyzing 40–100 glomeruli using QuPath. **** $p < 0.0001$ analyzed by Mann–Whitney U-test.

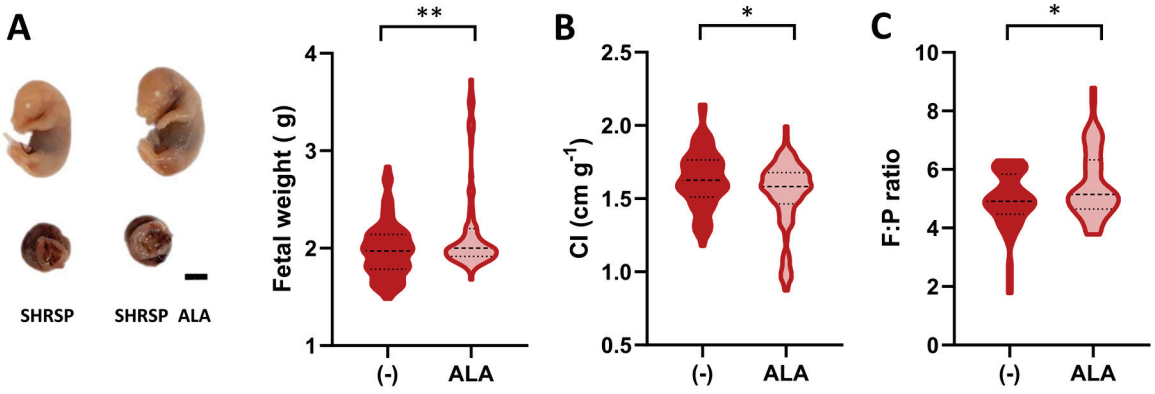


Figure 2. Effects of ALA supplementation on fetal phenotype. (**A**) Representative examples of formalin-fixed fetal and placental specimens collected on GD20. Scale bar: 5 mm. Fetal weight (violin plot) was significantly increased in the ALA-treated group. (**B**) Fetal cephalization indexes (CI, head circumference/body weight) measured on GD20. (**C**) Fetal to placental (F:P) weight ratio was measured in both treatment groups. Data in all panels are medians (dashed line) and minimum–maximum. (**A**,**B**): n = 88–85 specimens. (**C**): n = 53–54 specimens. ** $p < 0.01$, * $p < 0.05$ analyzed by Mann–Whitney U-test.

3.3. Treatment with ALA Attenuates Placental Oxidative Stress and NLRP3 Inflammasome Priming

Having observed an increased placental efficiency, we next hypothesized that the beneficial effect of ALA supplementation would be related to an improved placental environment in SHRSP pregnancies. Based on our previous findings showing an enhanced activation of oxidative stress pathways [19], we first evaluated the placental expression of NADPH oxidase (NOX) isoforms on GD14. ALA treatment led to a significant decrease in the expression of the cytosolic subunit NOXO1 (relative expression 1.00 ± 0.28 vs. 0.55 ± 0.08, $p = 0.0286$, Figure 3A) versus controls. However, we observed no differences in the expression levels of p47phox (relative expression 0.89 ± 0.29 vs. 0.93 ± 0.22, $p = 0.9762$, Figure 3A), NOX1, gp91phox ($p = 0.0952$ and $p = 0.2857$, respectively, Figure S3A) or the antioxidant SOD2 (relative expression 1.00 ± 0.19 vs. 0.85 ± 0.09, $p = 0.3429$, Figure 3B).

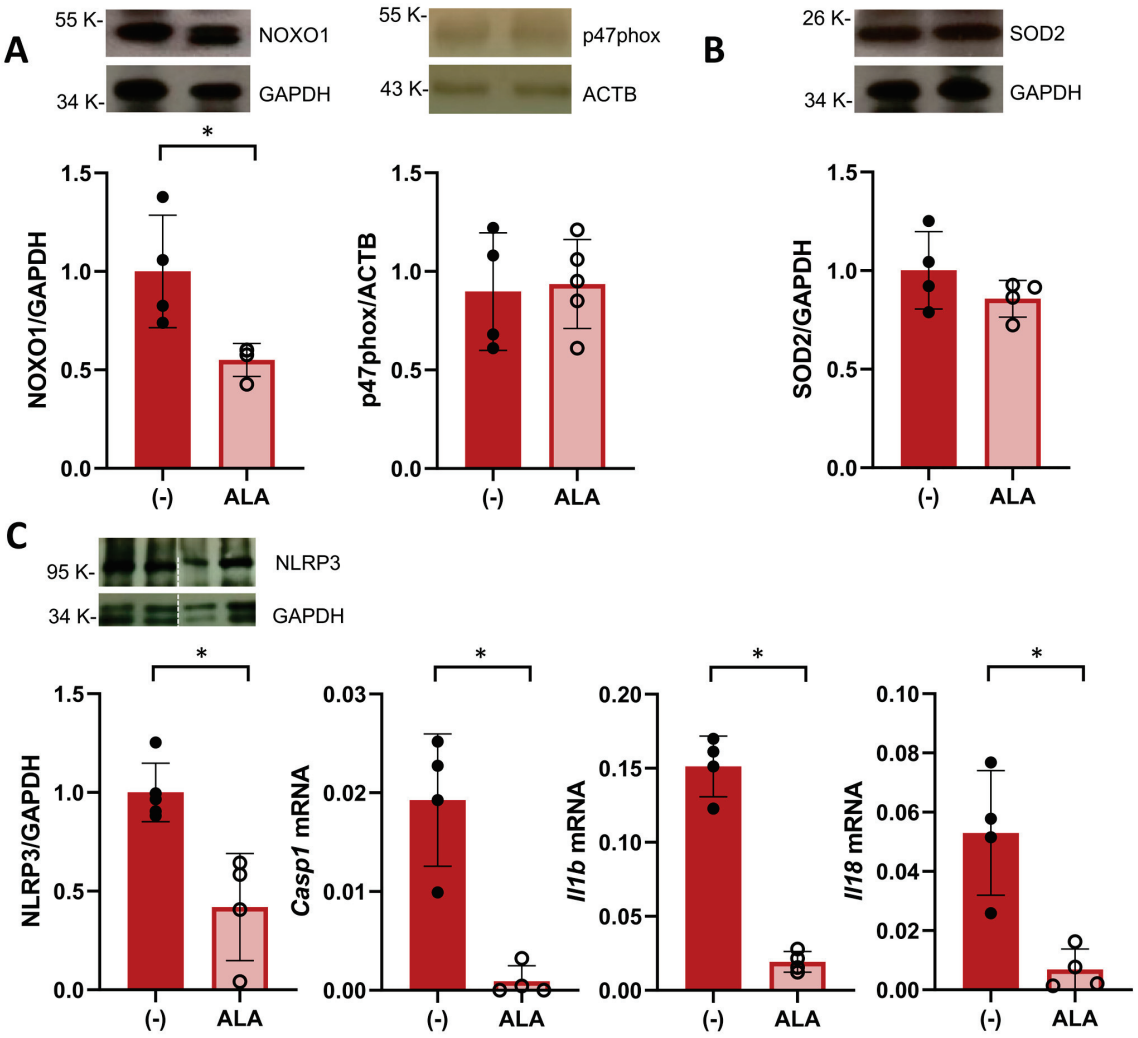


Figure 3. ALA attenuates placental oxidative stress and inflammasome priming. (**A**) Western blot analysis of placental expression of NOX subunits NOXO1 (left graph) and p47phox on GD14. Data are individual values and mean ± SD, $n = 4$–5 placentas/group, * $p < 0.05$ analyzed by Mann–Whitney U-test. (**B**) Placental levels of SOD2 analyzed on GD14. Data are individual values and mean ± SD, $n = 4$–5 placentas/group (**C**) Placental expression of components of the NLRP3 inflammasome pathway in ALA-treated SHRSP. On GD20, treated rats displayed decreased protein levels of NLRP3 (analyzed by western blot, left graph) and mRNA expression of caspase 1 (Casp1), IL1β (Il1b), and IL18 (Il18). Data are individual values and mean ± SD, $n = 4$–6 placentas/group, * $p < 0.05$ analyzed by Mann–Whitney U-test. Western blot and RT-PCR data are representative of 2 independent experiments run in duplicate.

Since NOX-derived ROS are known to promote NLRP3 inflammasome priming [28], we evaluated the expression of components of this pathway in the GD20 placenta. ALA treatment significantly decreased the protein expression of NLRP3 in SHRSP placentas (relative expression 1.00 ± 0.14 vs. 0.42 ± 0.27, $p = 0.0159$, Figure 3C) as well as decreased mRNA levels of caspase 1, IL1β and IL18 ($p = 0.0286$, $p = 0.0286$ and $p = 0.0286$, respectively, Figure 3C). Further analysis of amniotic fluid collected on GD20 revealed that ALA did not affect levels of thiobarbituric acid reactive substances (TBARS, $p = 0.5605$, Figure S3B) observed in SHRSP pregnancies but induced a marked decrease in IL18 protein levels (control 77.2 ± 26.8 pg/mg protein vs. ALA 32.0 ± 11.4 pg/mg protein, $p = 0.0068$, Figure S3C).

3.4. ALA Improves Placental Bed Vascular Remodeling and Labyrinth Vascularization in SHRSP Pregnancies

Next, we assessed the effect of ALA on placental dysfunction in the SHRSP model, which is characterized by defective spiral artery remodeling and impaired trophoblast differentiation associated with altered angiogenic signaling [18]. Histological evaluation of GD14 placentas showed that ALA treatment improved the physiological remodeling of spiral arteries, as shown by the significant loss of arterial wall ACTA2 expression in the mesometrial triangle of treated placentas (median expression control 63.12% vs. ALA 31.7%, $p < 0.0001$, Figure 4A left panels). Differentiation of the placental junctional zone was also restored in ALA-treated dams, denoted by the increased accumulation of PAS-positive glycogen trophoblasts compared to controls (median PAS-positive area control 29.29% vs. ALA 35.20%, $p = 0.015$, Figure 4A right panels). On GD20, treated dams displayed an increased vascular density in the placental labyrinth (median Isolectin B4(IB4)-positive staining control 26.04% vs. ALA 27.96%, $p = 0.0284$, Figure 4B). ALA decreased the placental mRNA expression of Flt1 and HIF1a ($p = 0.0286$ and $p = 0.0079$, respectively) but had no significant effect on levels of VEGF ($p = 0.2286$, Figure 4C). Compared to controls, serum levels of FLT1 on GD20 were significantly decreased in ALA-treated dams (control 120.0 ± 8.6 pg/mL vs. ALA 102.6 ± 11.3 pg/mL, $p = 0.0286$, Figure 4D).

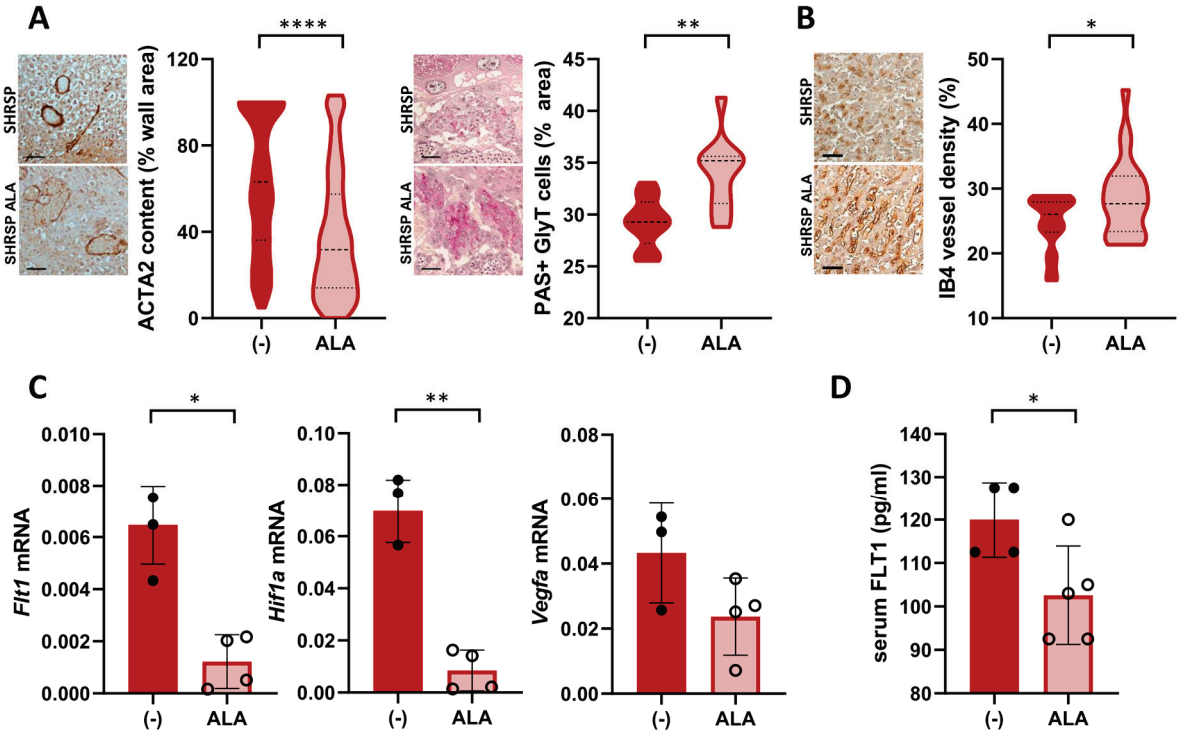


Figure 4. Treatment with ALA improves spiral artery remodeling, trophoblast differentiation, and angiogenic balance in SHRSP pregnancies. (**A**) Histological assessment of spiral artery remodeling

(αSMA staining, left graph) and glycogen trophoblast (GlyT) content (PAS-positive stain, right graph) in GD14 placenta. Scale bar: 50 μm. ALA restored arterial remodeling (noted by loss of arterial wall expression of ACTA2) and GlyT differentiation in treated dams. Data are medians (dashed line) and minimum–maximum, n = 6 samples/group analyzing > 100 artery cross-sections or 10–15 fields at ×200 using QuPath. **** $p < 0.0001$, ** $p < 0.01$ analyzed by t-test (**B**) Analysis of placental labyrinth vascularization on GD20, based on isolectin B4 (IB4) staining. Scale bar: 50 μm. Vascular density (%IB4-positive stain) was increased upon ALA treatment. Data are medians (dashed line) and minimum–maximum, n = 8–9 samples/group analyzing 20–30 fields at ×200 using Angiotool. * $p < 0.05$ analyzed by t-test. (**C**) Placental expression of Flt1 (Flt1, left graph), HIF1a (Hif1a, middle), and VEGF (Vegfa, right graph) analyzed by RT-PCR. Data are individual values and mean ± SD, n = 3–4 placentas/group, * $p < 0.05$, ** $p < 0.01$ analyzed by Mann–Whitney U-test. Results are representative of 2 independent experiments, run in duplicate. (**D**) Maternal serum FLT1 levels on GD20, determined by ELISA. Data are individual values and mean ± SD, n = 4–5 samples/group, * $p < 0.05$ analyzed by Mann–Whitney U-test.

4. Discussion

In this study, we demonstrated the protective effect of ALA supplementation in a rat model of hypertensive pregnancy with spontaneous progression to PE. In pregnant SHRSP, treatment with ALA during early pregnancy improved maternal blood pressure control and restored fetal growth associated with attenuated placental oxidative stress, inflammation, and antiangiogenic status. Given its beneficial effect on both maternal and fetal outcomes, ALA emerges as a promising therapeutic agent in pregnancies at high risk for PE.

The beneficial effects of ALA on cardiometabolic health, as well as its mechanisms of action, have been extensively studied [29,30]. Furthermore, evidence on the clinical use of ALA in pregnancy [31–33], including pharmacovigilance upon oral supplementation [34], has accumulated over the past decade, supporting its potential as a safe therapeutic intervention in PE. By showing an improved blood pressure regulation upon ALA treatment in pregnant rats with chronic hypertension, our preclinical findings are potentially translatable to a high-risk patient population that still imposes a challenge in terms of management. Current guidelines recommend antihypertensive therapy to limit the risk of progression to severe hypertension and low-dose aspirin and calcium for prevention of superimposed PE [35,36], but none of these strategies have proven definite in avoiding adverse maternal and perinatal outcomes. Antioxidant supplementation, in particular, has long been proposed as a therapeutic option for PE, but the many large-scale randomized clinical trials run so far have failed to demonstrate positive effects [11]. Our results in a preclinical model suggest that ALA may be successful where other antioxidants have failed, and this may be due to its diversity of molecular targets and inhibitory effects on cellular stress responses, inflammation, and endothelial dysfunction [12]. Regarding its effects on blood pressure, hypotensive effects upon ALA supplementation have been reported in animal studies [37,38] but are attained upon chronic treatment or single doses much higher than the one used in our study. Hence, the observed blood pressure-lowering response in SHRSP pregnancies may be secondary to the improved placental environment rather than a direct effect of ALA on the maternal vasculature. Collectively, these evidences encourage further investigation into the effect of ALA supplementation either alone or in combination with the currently recommended treatments in pregnant women with chronic hypertension.

A major finding in our study was the positive effect of ALA on placental insufficiency, which was noticed as early as GD14 through the improvement of spiral artery remodeling and differentiation of glycogen trophoblasts. In the SHRSP model, defective arterial remodeling [18] would result in intermittent high-pressure flow reaching the placenta, contributing to ischemia-reperfusion injury and ensuing oxidative stress. Sustained ROS production could then compromise cell organizational processes related to trophoblast

differentiation, resulting in the marked structural alterations observed both in the SHRSP and in the ischemic placenta of the reduced uteroplacental perfusion pressure (RUPP) PE model [39,40]. Hence, it is likely that the observed positive effect of ALA on placental insufficiency in our model is related to the multiple mechanisms by which it can prevent ROS accumulation [29], which have encouraged its therapeutic application against ischemia-reperfusion injury in both clinical and basic settings [41–43].

Further considering oxidative damage, glycogen trophoblasts seem to be particularly vulnerable [40] and display an impaired migratory potential in the ischemic RUPP placenta [39]. Glycogen trophoblast injury has major overall consequences in placental development and function since, at later stages, these cells invade the maternal compartment to further support arterial remodeling [44] and influence the differentiation of other placental lineages by producing retinoic acid [45]. Given that these cells are considered analogous to the human extravillous trophoblast [46], an intriguing question arising from our study is whether ALA can exert a direct influence on human trophoblast differentiation, migration, and invasive properties. Other than a study reporting an inhibitory effect of ALA on hypoxia-induced apoptosis of a villous trophoblast-derived cell line [47], evidence of its in vitro effects on human trophoblasts is still lacking.

During early pregnancy, placental ROS production occurs mainly through NOX activation and contributes to healthy trophoblast function by modulating redox-sensitive MAPK signaling pathways [48]. However, sustained activation of NOX plays a central pathogenic role in PE as it drives the placental secretion of sFlt1 [49] and may activate the NLRP3 inflammasome [28,50], promoting maternal systemic endothelial dysfunction and inflammation. Thus, the decreased placental expression of cytosolic NOXO1 (the organizer protein required for catalytic activation [51]) we observed upon ALA treatment suggests that inhibition of NOX-mediated ROS generation may be core to the protective effect of ALA in SHRSP pregnancies. Besides suppressing NOX activation, the effect of ALA on sFlt1 secretion could also be due to its potent ability to induce heme-oxygenase 1 activity [52], as this pathway has been shown to negatively regulate the release of sFlt1 and soluble endoglin from villous explants [53]. Indeed, considering the treatment timeframe in our study, it is likely that the antioxidant effects of ALA improved the physiological adaptation of the SHRSP placenta to the oxidative burst associated with the onset of maternal blood flow to the hemochorial interface, which in the rat occurs around GD12 [54]. This is highlighted by the finding that the inhibitory effects we observed on sFlt1, IL1β, and IL18 persisted on GD20 beyond the temporal window of ALA administration.

Besides mitigating maternal signs, it is desirable that a PE therapeutic agent would prevent adverse fetal outcomes associated with FGR. Here, we demonstrated the ability of ALA to rescue asymmetric FGR in the SHRSP model, which is consistent with previous animal studies reporting similar effects in models of diabetic [55] and toxicant-induced intrauterine growth restriction [56]. Asymmetric FGR, as observed in the SHRSP [18,27], results from impaired nutrient transfer due to placental dysfunction and thus shows a strong association with PE [57]. The protective effect of ALA on fetal growth likely stems from improved placental efficiency for metabolic exchange, as suggested by the increased fetal-to-placental weight ratios and enhanced labyrinth vascularization observed upon ALA treatment. However, an interesting finding of our study is that ALA treatment was associated with decreased levels of IL18 in the amniotic fluid, suggesting that the anti-inflammatory effects of ALA can also reach the fetal compartment via maternal supplementation. Since growth factors and signaling molecules in the amniotic fluid can be absorbed by the fetus and potentially influence its development [58], our study opens an exciting avenue of research into the developmental programming effects induced by maternal ALA supplementation. Recent studies, for instance, have demonstrated that maternal ALA treatment can attenuate the metabolic alterations associated with the consumption of high-fructose diets in rat offspring [59], supporting a role for ALA in programming cardiometabolic health in utero.

Translational research remains a challenge for both bench scientists and physicians, particularly in obstetrics, due to concerns related to safety and teratogenicity. While further research involving larger cohorts should be encouraged, a retrospective study of 610 pregnancies reported no adverse effects in the mother or newborn upon daily oral administration of 600 mg ALA for at least seven weeks [34], opening a reassuring scenario regarding its therapeutic use during pregnancy. Based on an allometric scaling approach for interspecies dose conversion [60], 600 mg represents a dose higher than the one administered in our study, adding to the clinical relevance of our findings. However, there are limitations inherent to the animal model that need to be taken into account when extrapolating our results. The main consideration is that unlike in PE subjects, blood pressure in hypertensive rat models decreases towards delivery, as reported in the SHRSP and other strains [61]. Furthermore, we acknowledge that no single animal model of PE can fully replicate the human syndrome due to disease heterogeneity, diversity of molecular signatures, clinical manifestations, and outcomes [62]. However, the pregnant SHRSP phenotype not only fulfills the current definition of PE, combining hypertension with end-organ damage or FGR [2], but also presents several important traits associated with PE pathogenesis, including defective spiral remodeling, placental dysfunction, and angiogenic imbalance. Given its effects in mitigating maternal signs and restoring placental function in this model, we propose that ALA supplementation represents a promising intervention to improve the outcomes of pregnancies at high risk for PE, for which there is currently no definite therapeutic option.

5. Conclusions

In conclusion, ALA supplementation mitigated maternal signs and rescued the placental dysfunction and FGR associated with the development of PE in our preclinical model of hypertensive pregnancy. Our preclinical observations suggest that ALA could be successful where other antioxidants have failed and encourage further investigation into the effect of ALA supplementation on pregnant women with chronic hypertension, either alone or in combination with the currently recommended treatments. Further studies should address the therapeutic use of ALA in other preclinical models of PE or isolated FGR, as well as examine the effects on human trophoblast biology in vitro. This knowledge, together with accumulating clinical evidence on the safety of ALA administration in pregnancy, set the ground for further studies aimed at repositioning ALA as a preventive therapy for pregnancies at high risk for PE.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antiox13060730/s1. Figure S1: Effect of ALA supplementation on maternal signs; Figure S2: Effect of ALA supplementation on fetal phenotype; Figure S3: Analysis of oxidative stress and inflammation markers in placenta and amniotic fluid; Expanded Materials and Methods (including Tables S1–S4); Full unedited blots for Figure 3; Supporting Data Values file.

Author Contributions: Conceptualization, G.B.; methodology, G.B., M.G. and S.I.G.; formal analysis, G.B., M.G. and S.I.G.; investigation, G.B., M.L.S., M.S.L., E.R., M.G. and S.I.G.; resources, G.B., C.I., M.L.C., M.G. and S.I.G.; writing—original draft preparation, G.B.; writing—review and editing, G.B. and M.L.C.; supervision, G.B.; funding acquisition, G.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care Committee (CICUAL, Facultad de Medicina, Universidad de Buenos Aires, License 2064/2015).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Transwell Culture with Adipose Tissue-Derived Stem Cells and Fertilized Eggs Mimics the In Vivo Development of Fertilized Eggs to Blastocysts in the Fallopian Tube: An Animal Study

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Abstract: Many countries, including Japan, are experiencing declining birth rates. Assisted reproductive technologies have consistently demonstrated good results in resolving infertility. Although the development of fertilized eggs into blastocysts has been recognized as a crucial step in assisted reproductive technologies, the involved mechanisms are currently unclear. Here, we established a new culture system for the in vitro development of fertilized eggs into blastocysts. In the Transwell culture system, the rate of blastocysts hatching from fertilized eggs cultured with adipose-derived stem cells (ASCs) was significantly higher than that of blastocysts cultured only with fertilized eggs. Gene ontology analysis revealed that the developed blastocysts displayed essential gene expression patterns in mature blastocysts. Additionally, when cultured with 3rd-passage ASCs, the developed blastocysts expressed the core genes for blastocyst maturation and antioxidant properties compared to those cultured only with fertilized eggs or cultured with 20th-passage ASCs. These results suggest that the Transwell culture system may imitate the in vivo tubal culture state for fertilized eggs. Exosomes derived from stem cells with stemness potential play a powerful role in the development of blastocysts from fertilized eggs. Additionally, the exosomes expressed specific microRNAs; therefore, the Transwell culture system resulted in a higher rate of pregnancy. In future, the extraction of their own extracellular vesicles from the culture medium might contribute to the development of novel assisted reproductive technologies.

Keywords: blastocyst; extracellular vesicles; adipose stem cells; stemness; Transwell culture

1. Introduction

Approximately 15–20% of couples worldwide have been diagnosed with infertility or sterility, which is defined as the failure to conceive despite ordinary sexual intercourse without contraception [1]. Assisted reproductive technologies (ARTs) have contributed to solving medical, economic, and social issues related to infertility [2]. The use of ARTs to overcome infertility has been associated with improvements in the in vitro environment for embryo production [3]. Although various molecules in the female reproductive system are involved in the equilibrium between oxidants and antioxidants, the risk of oxidative damage is possibly magnified in an in vitro system of ART [4]. ARTs involve many artificial

procedures that may cause damage, such as oxidative stress [5]. Consequently, many reactive oxygen species (ROS) are generated and accumulate in in vitro-produced embryos during the process of ART. Oxidative damage results in the impairment of blastocyst development and hatching, leading to a decrease in the fertilization rate [6–8]. Accumulating evidence has revealed that antioxidants play a pivotal role in reducing oxidative damage in assisted reproductive technologies, particularly in in vitro-produced embryos [9]. The addition of antioxidants activates the development of embryos during the preimplantation process. Many studies have shown that the development of blastocysts from fertilized eggs, as well as the preimplantation of blastocytes, can be improved by the addition of antioxidant substances to the culture medium of fertilized eggs [10–13]. Recent studies have indicated that mesenchymal stem cells possess antioxidant properties against biological events [14]. Furthermore, a few studies have reported that mesenchymal stem cells are associated with the development of blastocysts in in vitro-produced embryos, suggesting that certain substances secreted from mesenchymal stem cells may play a critical role in the development of blastocysts [8,15]. In principle, stem cells can self-renew and differentiate into a variety of specialized cell types possessing infinite regenerative potential through their trophic properties [16–20]. Stem cells originate from four main sources: embryonic, fetal, adult, and differentiated somatic cells [21]. Mesenchymal stem cells isolated from differentiated somatic cells have been used in regenerative medicine [22]. They also play an influential role in the regeneration of damaged cells and tissues in various diseases by secreting paracrine factors and extracellular vesicles (EVs) [23]. To date, EVs secreted from stem cells have attracted considerable attention due to their ability to retain stem cell stemness. Adipose-derived mesenchymal stem cells (ASCs) are commonly used in various clinical fields because of their strong antioxidant properties [24–27]. Among the different types of MSCs, ASCs are unique owing to their minimally invasive harvesting procedure, capacity for efficient bulk retrieval, and commendable cell proliferation rates [28]. In addition, EVs derived from mesenchymal stem cells have been recognized as promising therapeutic agents for various disorders, serving as antioxidant molecules [29,30]. Any cell type can secrete EVs, both in physiological and pathological conditions. In principle, EVs can be broadly categorized into exosomes and ectosomes [31]. Ectosomes, which pinch off the surface of the plasma membrane via outward budding, consist of microvesicles, microparticles, and large vesicles with diameters of 50–1000 nm. Exosomes, which originate from endosomes, range from 40 to 160 nm in diameter. EVs contain many cellular components, including microRNAs, RNA, DNA, lipids, metabolites, cytokines, and proteins. Recently, EVs have emerged as an attractive research field because of their central role in intercellular communication. In female reproduction, EVs communicate important information between cells and tissues during folliculogenesis, fertilization, embryo formation, and implantation [32–35]. EVs derived from oviduct fluid may enhance embryo development [33]. However, it is not clear whether this positive effect can be attributed to these EVs. To date, a few reports have described that mesenchymal stem cells stimulate the development of blastocysts in in vitro-produced embryos, suggesting that a variety of paracrine factors as well as EVs may participate in the development of blastocysts in in vitro-produced embryos [8,15]. In this study, we aimed to examine the development of blastocysts from in vitro-produced embryos to create a new Transwell culture system for in vitro fertilization. In addition, we aimed to identify the paracrine factors and EVs secreted by mesenchymal stem cells that are involved in the development of blastocysts in in vitro-produced embryos. Embryos developed in Transwell culture showed enhanced expression of genes involved in cytoskeleton, pluripotency, and embryonic organ development. Furthermore, EVs derived from 3rd-passage stem cells possessed much more potential than those derived from 20th-passage stem cells did. EVs derived from 3rd-passaged stem cells contained various specific miRNAs.

2. Materials and Methods

2.1. Cell Culture

In a Transwell culture system, two different cell types can be cultured separately in each medium. By utilizing a 400 nm pore filter, specific substances present in the medium of the upper layer can be transferred to the medium of the lower layer.

2.2. Animals

Twelve-week-old ICR female mice were purchased from KBT Oriental (Saga, Japan) to obtain adipose tissue-derived stem cells (ASCs). Frozen 2-cell embryos were purchased from the Biosafety Research Center, Inc. (Hyogo, Japan). All animal experiments were approved by the Animal Experiment Committee of Fukuoka University (approval No. 1915132).

2.3. Isolation of ASCs

ASC isolation was conducted in accordance with a previously documented protocol [36]. In brief, subcutaneous adipose tissue specimens were obtained from ICR mice to procure adipose-derived regenerative cells (ADRCs). The ADRCs were cultivated in a MEMα/GlutaMax medium (Gibco, Grand Island, NY, USA). Moreover, they were supplemented with 10% Exo-FBS (System Biosciences, Palo Alto, CA, USA) and a 1% antibiotic–antimycotic solution (Gibco) within a humidified incubator set at 37 °C with 5% CO_2. For experimental purposes, ASCs from early or late passages (3rd or 20th passages, respectively) were utilized.

2.4. In Vitro Culture of Embryos

We divided the upper and lower chambers and used Transwell filters (Greiner Bio-One, 24-well, pore size 0.4 μm) for nanoparticle transport studies. ASCs were seeded into the upper chamber at a density of 1×10^5 cells/well as superscript with 100 μL of MEMα/GlutaMax medium containing 10% Exo-FBS and 1% antibiotic–antimycotic solution. Thereafter, frozen 2-cell embryos were incubated in the lower chamber filled with 600 μL KSOM at 37 °C and 5% CO_2 on Day 0 (Figure 1A). Both chambers underwent medium exchange on Day 2. We observed blastocyst formation and hatching on Day 4. Frozen 2-cell embryos incubated with KSOM without the Transwell filter were used as controls. No cells were observed in the upper chamber, which was defined as the T-control group. Early passage ASCs and late passage ASCs seeded in the upper chamber were defined as E-ASC and L-ASC groups, respectively (Figure 1A). Morphological evaluation of the mouse embryos was performed stepwise from the 2-cell stage to hatching.

2.5. Flow Cytometric Analyses

Flow cytometric analyses of ASCs were performed according to a previously reported protocol [36]. In brief, ASCs were suspended in a staining buffer (R&D Systems, Minneapolis, MN, USA). Following this, they were incubated with CD105, CD29, Sca-1, and CD45 (all from the Mouse Mesenchymal Stem Cell [MSC] Multi-Color Flow Kit, cat. no. FMC003; R&D Systems). Flow cytometry was performed using FACSAria™ (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell culture supernatant interleukin (IL)-6, vascular endothelial growth factor (VEGF)-A, leptin, adiponectin, and fibroblast growth factor (FGF)2 levels were determined by ELISA, following the manufacturer's instructions. Mouse IL-6, VEGF-A, leptin, adiponectin, and FGF2 ELISA kits were purchased from Proteintech (Rosemont, IL, USA).

2.7. In Vitro Culture of Embryos with Antibody

Mouse VEGF Antibody (#AF-493-NA), Mouse IL-6 Antibody (#MAB406-100), Normal Rat IgG Control (#6-001-A), and Normal Goat IgG Control (#AB-108-Cmouse) were pur-

chased from R&D Systems (Minneapolis, MN, USA). In a Transwell culture system with E-ASCs, these antibodies were suspended at 1 µg/mL in the KSOM at the lower chamber. We observed blastocyst formation and hatching on Day 4.

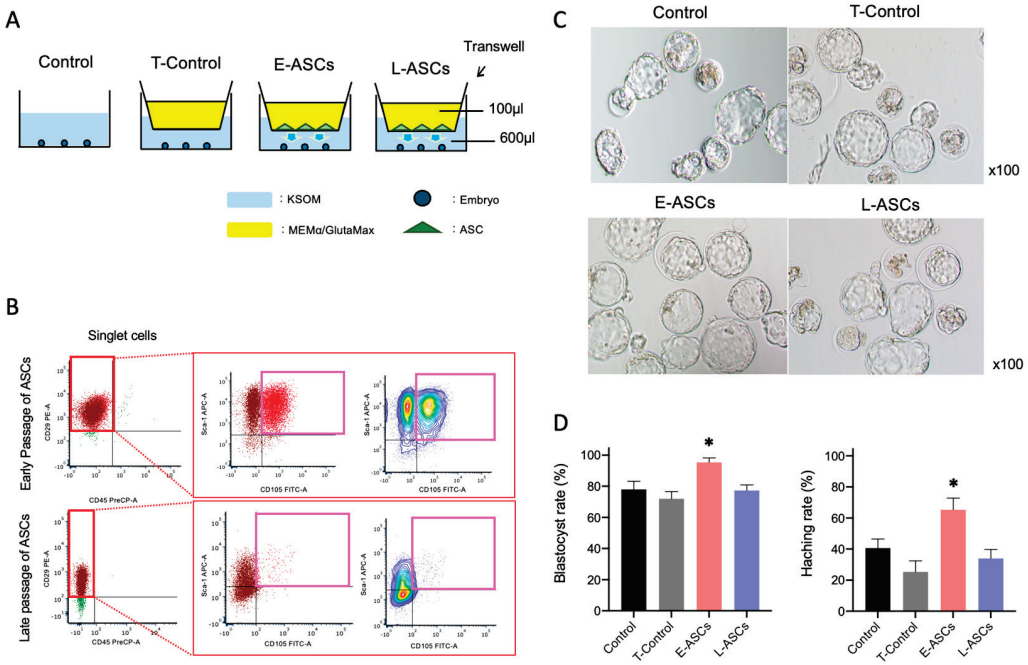


Figure 1. (**A**) Scheme of in vitro culture of embryos using a Transwell. Control: embryos incubated KSOM without Transwell; T-Control: no cells in the upper chamber; E-ASCs (adipose tissue-derived mesenchymal stem cells): early passage of ASCs (P3: 3rd passage) seeded in the upper chamber; L-ASCs: late passage of ASCs (P20: 20th passage) seeded in the upper chamber. (**B**) Multicolor flow cytometry analysis of cells in the plot of singlet cells based on staining for CD105, CD29, Sca-1, and CD45. The percentages of mesenchymal stem cells, including early passage (3rd passage) adipose tissue-derived stem cells (ASCs) and late passage (20th passage) ASCs, harboring the cell-surface expression markers CD29, Sca-1, and CD105, and negative CD45 are shown. Cells in pink box indicate mesenchymal stem cells. (**C**) Embryo morphology on Day 4. (**D**) Blastocyst and hatching rates of in vitro-produced mouse embryos co-cultured with ASCs. The mean percentage was calculated from three independent experiments. n = 150 per group. Data are presented as the mean ± standard error. * $p < 0.05$ vs. Control.

2.8. Generation of Stably Transfected ASCs

Short interfering RNAs (siRNAs) of the Universal Negative Control (#SIC001), IL-6 (#EMU011231), and VEGFA (#EMU062931) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). The siRNAs were transfected into E-ASCs using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, the transfected E-ASCs were used for the in vitro culture of embryos using a Transwell. We observed blastocyst formation and hatching on Day 4. Supernatants in the lower chamber were harvested, and the protein levels of IL-6 and VEGFA were evaluated using an ELISA.

2.9. Ultracentrifugation to Isolate EVs

We isolated EVs from the supernatants of ASCs by ultracentrifugation, as described previously [37]. The supernatant from the lower chamber was harvested and centrifuged using a KUBOTA Centrifuge (Tokyo, Japan) at 300× g at 4 °C for 10 min to remove detached cells. Next, the supernatant was collected and centrifuged at 2000× g at 4 °C for 10 min to remove detached cells. Furthermore, the supernatant was collected and centrifuged at 10,000× g at 4 °C for 30 min to remove contaminating apoptotic bodies, microvesicles, and cell debris. Clarified cell culture medium was then centrifuged in a Beckman Coulter

Optima™ MAX- Ultracentrifuge at 100,000× g_{avg} at 4 °C for 70 min with a Type MLS-50 rotor (k-factor: 71) to pellet the exosomes. The supernatant was removed carefully, and the crude exosome-containing pellets were resuspended in 1 mL of ice-cold phosphate-buffered saline (PBS) and pooled. A second round of ultracentrifugation [100,000× g_{avg} at 4 °C for 70 min with a Type MLS-50 rotor (k-factor: 71)] was carried out, and the resulting exosome pellet was resuspended in proper culture medium.

2.10. Nanoparticle Tracking Analysis (NTA) Measurement

Supernatants in the lower chamber were harvested, and nuclei and cell debris were removed by centrifugation. The number of nanoparticles (NPs) in the supernatant was measured by NTA using a NanoSight LM10 (NanoSight, Malvern, UK). The ideal measurement concentrations were determined by pretesting the ideal particle-per-frame value (20–100 particles/frame). The following settings were set according to the manufacturer's software manual (NanoSight LM10 User Manual, MAN0510-04-EN-00). The camera level was adjusted to level 8 until all particles were distinctly visible and did not exceed a particle signal saturation of over 20%. The autofocus was adjusted to avoid indistinct particles. After capturing, the videos were analyzed using NanoSight Software NTA 3.0.

2.11. Western Blot Analysis

EV pellets obtained by ultracentrifugation were resuspended in RIPA Lysis and Extraction Buffer (#89900; ThermoFisher Scientific, Tokyo, Japan) supplemented with Protease Inhibitor Cocktail (#78410; ThermoFisher Scientific) and Phosphatase Inhibitor Cocktail (#78420; ThermoFisher Scientific). The total proteins of EVs were resolved by 10% precast SDS/PAGE gels (#195-14951; FUJIFILM Wako, Osaka, Japan) and transferred to a Poly Vinylidene DiFluoride (PVDF) membrane using iBlot2 (ThermoFisher Scientific). The membrane was blocked with PVDF Blocking Reagent for Can Get Signal® (TOYOBO, Osaka, Japan) for 1 h at room temperature under constant shaking. Subsequently, the membranes were incubated with rabbit polyclonal CD63 (1:1000, Proteintech), CD9 (1:1000, Cell Signaling Technology, Danvers, MA, USA), and CD81 (1:1000, Cell Signaling Technology) antibodies overnight at 4 °C. Membranes were washed three times with 1× Tris-buffered saline containing Tween 20 (TBS-T) and then incubated with rabbit horseradish peroxidase-conjugated secondary antibodies (#7074S; Cell Signaling Technology; 1:1000 dilution) in a blocking solution for 1 h at room temperature under constant shaking. After washing the membranes thrice with TBS-T, the blots were developed using Immobilon Western Chemiluminescent HRP Substrate (#61-0205-40; Merck Millipore, Burlington, MA, USA) and visualized using an Amersham™ Imager 680 Imaging System (Cytiva, Tokyo, Japan) and Amersham™ Imager 680 Analysis Software Version 2.0 (Cytiva, Tokyo, Japan).

2.12. Extracellular Nanovesicles Labeling and Uptake

The EVs obtained via ultracentrifugation were labeled using the ExoGlow-Membrane EV Labeling Kit (Cat # EXOGP400A-1, System Biosciences) as per the manufacturer's protocol. Following labeling, the EVs were suspended in KSOM and introduced to morulae, which were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Prior to cell treatment, all samples underwent ultracentrifugation. Moreover, any residual dye from the exosome labeling process was eliminated using a PD MiniTrap G-25 (Cytiva) in accordance with the manufacturer's instructions. Subsequently, the labeled exosomes were administered after a 30 min incubation with Pitstop2. Following a 24 h incubation period, the uptake process was halted by the washing and fixation of the cells in a 3.7% PFA solution for 10 min. Subsequent staining with DAPI allowed for the visualization of the cells under a fluorescence microscope (Keyence, Osaka, Japan).

2.13. Library Preparation and Sequencing of EV miRNAs

To obtain the supernatant of E-ACSs or L-ASCs, ASCs were incubated on a 100 mm plate in 10 mL of MEMα/GlutaMax medium containing 10% Exo-FBS and 1% antibiotic-

antimycotic at 37 °C and 5% CO_2. The supernatant was collected after 48 h and filtered using a 0.22 µm filter to remove detached cells and debris (N = 3). For miRNA-seq, miRNAs were obtained from the supernatant using the exoRNeasy Serum/Plasma Maxi Kit (QIAGEN). Sequencing libraries were constructed using a QIAseq miRNA Library Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Library quality was assessed using the Agilent 2100 Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). The pooled libraries of the samples were sequenced using NextSeq 500 (Illumina, Inc., San Diego, CA, USA) in 76 base-pair (bp) single-end reads. Raw data were applied to the bioinformatics pipeline, which included quality control with FastQC ver.0.12.1 and MultiQC ver.1.18.

2.14. Quantifying the miRNA Expression Level and Detection of Differentially Expressed Genes

The QIAseq miRNA Library Kit adopts a unique molecular indices (UMIs) system, enabling the unbiased and accurate quantification of mature miRNAs. All reads assigned to a particular miRNA (miRBase Release 22) were counted, and the associated UMIs were aggregated to count unique molecules using CLC Genomics Workbench 23.0.2 (QIAGEN, Valencia, CA, USA). The UMI counts of miRNAs were normalized using CPM (TMM-adjusted), which stands for Trimmed Mean of M values adjusted Counts Per Million [38], in the CLC Genomics Workbench. Normalized values were subjected to downstream analyses using StrandNGS 4.0 software (Agilent Technologies, Santa Clara, CA, USA). Statistical analysis between two groups ("sample" and "control") was performed by moderated *t*-test [39]. Differentially expressed genes (DEGs) were considered significant if the Fold Change (FC) was >1.5.

2.15. cDNA Library Preparation and RNA Sequencing of Blastocysts

Blastocyst samples were collected from the control, E-ASC, and L-ASC groups on Day 4 (N = 8 for each group). Sequencing libraries were prepared for each sample by using NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina® (New England BioLabs, Ipswich, MA, USA) and NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) (New England BioLabs) in accordance with the manufacturer's instructions (Illumina, San Diego, CA, USA). Library quality was assessed using the Agilent 2100 Bioanalyzer/High Sensitivity (Agilent Technologies). The libraries were sequenced using Illumina's sequencer HiSeq X (Illumina, San Diego, CA, USA) with paired-end 151 bp and dual index settings.

2.16. RNA-Sequencing Data Analysis of Blastocysts

Raw sequencing reads of 24 RNA sequencing libraries, ranging from 15.0 to 22.4 million read pairs, were subjected to transcript quantification using Salmon 1.5.2 (https://salmon.readthedocs.io/en/latest/salmon.html, accessed on 23 July 2021) [40]. To run the mapping-based mode for salmon, a salmon transcriptome index was generated for gencode.vM23.transcripts.fa.gz using the entire human genome (GRCm38.primary_assembly.genome.fa.gz) as the decoy sequence. The resultant quant.sf data of the 24 samples were integrated into one file using the R package tximport 1.20.0 [41]. The resultant count data for 54,281 gene IDs were subjected to further analyses using an R package, EdgeR 3.36.0 [42], such as filtering, normalization, clustering analyses, and the identification of DEGs between two conditions (FDR q-value < 0.0.5 as a threshold). Gene ontology analyses of the DEGs were conducted using Metascape 3.5 [43].

2.17. Statistical Analysis

All data were analyzed using the Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). The results were shown as mean ± standard error. Data regarding blastocyst formation rates, hatching rates, cytokine concentrations, and nanoparticle concentrations among the different groups were analyzed using the Mann–Whitney U test and Benjamini–Hochberg method. p values were considered significant when they were less than 0.05.

3. Results

3.1. Cellular Characteristics of ASCs

To examine the expression of cell-surface markers of MSCs, flow cytometry was performed using antibodies against CD105, CD29, and Sca-1 as positive markers and CD45 as a negative marker. For cells in the P1, P2, and P3 plots, the phenotype was analyzed based on stained cell surface markers (Figure 1B). The percentage of MSCs in ASCs was 48.6 ± 4.3% (n = 3) at the early cell passage (passage 3) (Figure 1B), which decreased to 2.1 ± 0.4% (n = 3) by the late cell passage (passage 20) (Figure 1B). These results suggested that serial subcultivation induced a marked decrease in ASC stemness.

3.2. Effect of ASCs on Embryo Development

In the control group, 78.0 ± 7.5% of 2-cell embryos developed into a blastocyst on Day 4. In contrast, 95.3 ± 4.1% of the E-ASCs group developed into a blastocyst, and 25.3 ± 10.0% hatched (Figure 1C,D). In the E-ASC group, the embryo development and hatching rates were significantly higher than those in the control group ($p < 0.05$). However, no significant differences in development or hatching rates were observed among the control, T-control, and L-ASC groups (Figure 1D). These results indicate that the co-culture of E-ASCs with embryos using Transwell increased embryo development.

3.3. Effect of Cytokines on Embryo Development

In the E-ASC groups, IL-6 and VEGFA protein levels were significantly higher than those in the control and L-ASC groups ($p < 0.05$). Only the VEGFA protein levels of the L-ASC group were significantly higher than those of the control group ($p < 0.05$). No significant differences were detected in the protein levels of FGF2, leptin, and adiponectin among the T-control, E-ASC, and L-ASC groups (Figure 2A).

Blastocyst development and hatching rates did not decrease, even when anti-IL-6 antibodies or anti-VEGFA antibodies were added to the culture medium (Figure 2B).

In addition, the blastocyst differentiation ability was not reduced by E-ASC-transfected siRNA, which inhibited IL-6 and VEGFA secretion (Figure 2C).

3.4. Comparison of Exosome Abundance and miRNAs Contained by Different ASC Passages

EVs, including exosomes, shedding vesicles, prostasomes, and apoptotic bodies, are membrane vesicles of 40–1000 nm that are released from many cell types, including red blood cells, platelets, lymphocytes, dendritic cells, endothelial cells, and tumor cells [44]. In particular, CD9, CD63, and CD81 are recognized as exosome-specific markers [45]. In this study, the size of the nanoparticles ranged between 50 and 600 nm, and most of them were between 100 and 200 nm in all media groups. The numbers of nanoparticles were $8.2 \times 10^8 \pm 3.51 \times 10^7$/mL in the T-Control group, $2.74 \times 10^9 \pm 1.90 \times 10^8$/mL in the E-ASC group, and $1.98 \times 10^9 \pm 2.75 \times 10^8$/mL in the L-ASC group (Figure 3A). These nanoparticles expressed CD63, CD81, and CD9, as determined by Western blotting, and were presumed to be exosomes (Figure 3B). From the difference in the number of nanoparticles, the numbers of exosomes in E-ASCs (E-EVs) and L-ASCs (L-EVs) were estimated at approximately 1.92×10^9/mL and 1.16×10^9/mL, respectively. Expression analysis of microRNAs in EVs derived from E-ASCs and L-ASCs utilizing a next-generation sequencing technique unveiled an upregulation of miR-141-3p, miR-676-3p, miR-181a-2-3p, miR-429-3p, miR-200c-3p, miR-379-5p, miR-154-5p, miR-200b-3p, miR-329-3p, miR-497a-5p, miR-149-5p, miR-382-5p, miR-665-3p, miR-132-3p, miR-431-3p, miR-127-3p, miR-21a-5p, miR-376b-3p, miR-206-3p, and miR-370-3p (Figure 3C). These findings suggest that while both E-ASCs and L-ASCs release EVs, E-ASCs demonstrate a greater abundance and a distinct repertoire of miRNAs.

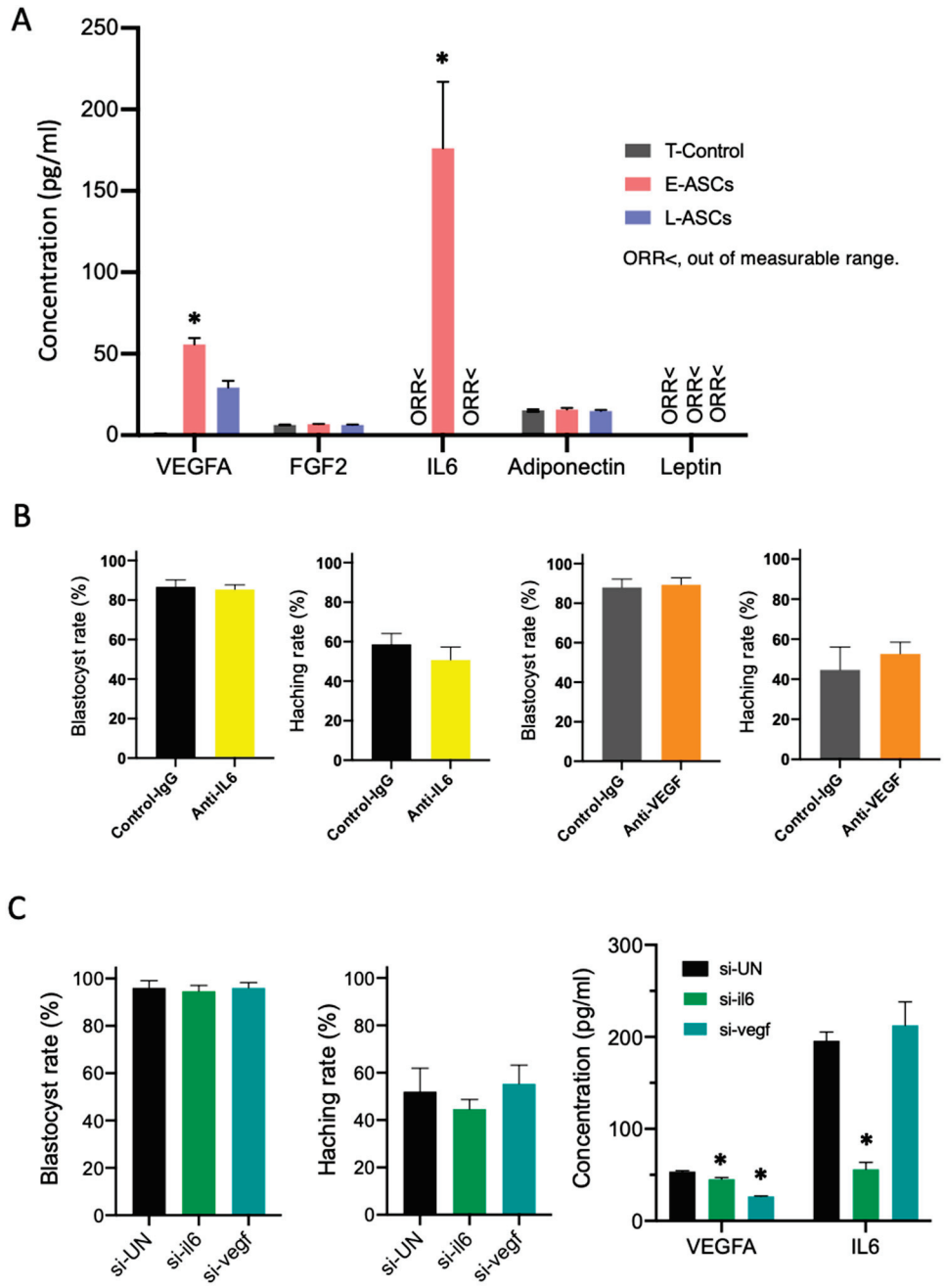


Figure 2. (**A**) Factor composition of the lower chamber. T-Control: no cells in the upper chamber; E-ASCs (adipose tissue-derived mesenchymal stem cells): the early passage of ASCs (P3: 3rd passage) seeded in the upper chamber; L-ASCs: the late passage of ASCs (P20: 20th passage) seeded in the upper chamber. Data are presented as the mean ± standard error. * $p < 0.05$ vs. T-Control. <ORR: out of measurable range. (**B**) Blastocyst and hatching rates for in vitro culture of embryos with mouse VEGF antibody or mouse IL-6 antibody. (**C**) Blastocyst rates, hatching rates, and concentration of VEGFA and IL-6 for in vitro culture of embryos with E-ASCs transfected with short interfering RNA (siRNA). Data are presented as the mean ± standard error. * $p < 0.05$ vs. Control. UN: Universal control.

3.5. Effect of Exosomes on Embryo Development

The E-ASC-EVs fluorescently labeled red by the ExoGlow-Membrane EV Labeling Kit were observed to be integrated into the inner blastocyst, as verified through fluorescence microscopy. On the other hand, the inhibition of E-ASC-EV uptake by pitstop2 was similarly confirmed by fluorescence microscopy (Figure 3D). The blastocyst development and hatching rates when incubated in KSOM mixed with E-ASCs-EVs (94.0 ± 4.3% and 72.7 ± 11.6%, respectively) were higher than those of blastocysts with inhibited E-ASCs-EVs uptake by pitstop2 (77.3 ± 4.1% and 37.3 ± 11.6%, respectively) (Figure 3E). However, L-ASC-EVs did not improve the blastocyst formation rate (Figure 3E).

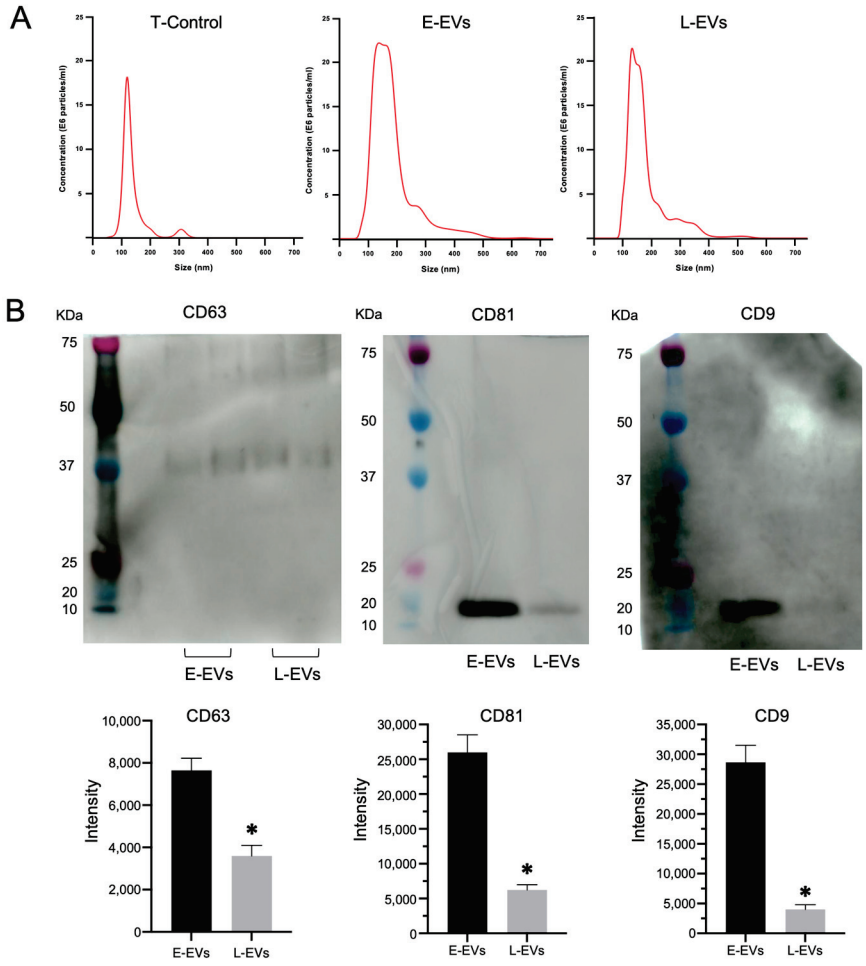
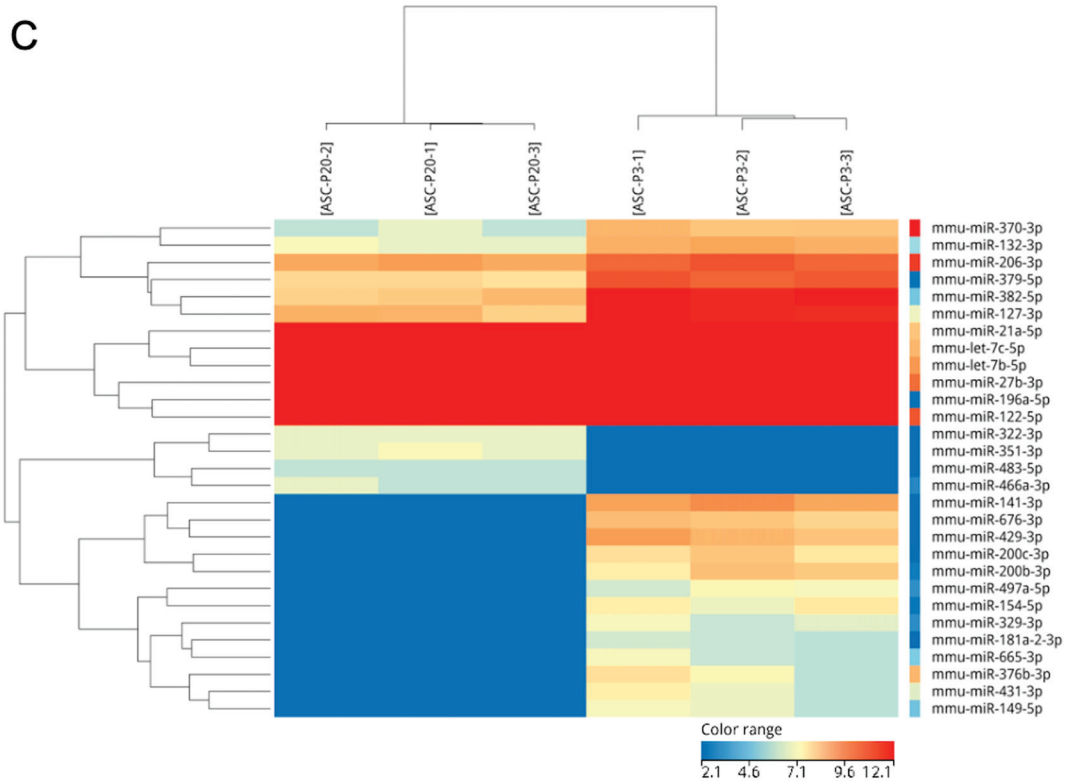


Figure 3. *Cont.*



Gene ID	p (Corr)	p	FC (abs)	FC	Log FC	Identifier
mmu-miR-141-3p	8.89×10^{-5}	1.36×10^{-7}	903.95	903.95	9.82	MI0000166
mmu-miR-676-3p	2.88×10^{-4}	5.87×10^{-7}	414.67	414.67	8.70	MI0005003
mmu-miR-181a-2-3p	3.38×10^{-4}	8.62×10^{-7}	54.19	54.19	5.76	MI0000223
mmu-miR-429-3p	5.46×10^{-4}	1.95×10^{-6}	625.82	625.82	9.29	MI0001642
mmu-miR-200c-3p	7.12×10^{-4}	2.91×10^{-6}	294.20	294.20	8.20	MI0000694
mmu-miR-379-5p	0.00117562	6.00×10^{-6}	7.79	7.79	2.96	MI0000796
mmu-miR-154-5p	0.00148026	8.31×10^{-6}	168.39	168.39	7.40	MI0000176
mmu-miR-200b-3p	0.00198192	1.21×10^{-5}	338.18	338.18	8.40	MI0000243
mmu-miR-329-3p	0.00363386	2.60×10^{-5}	86.03	86.03	6.43	MI0000605
mmu-miR-497a-5p	0.00393336	3.01×10^{-5}	112.73	112.73	6.82	MI0004636
mmu-miR-149-5p	0.00916448	7.48×10^{-5}	86.03	86.03	6.43	MI0000171
mmu-miR-382-5p	0.00961642	8.34×10^{-5}	11.54	11.54	3.53	MI0000799
mmu-miR-665-3p	0.01037024	9.52×10^{-5}	68.28	68.28	6.09	MI0004171
mmu-miR-132-3p	0.01310798	1.27×10^{-4}	6.07	6.07	2.60	MI0000158
mmu-miR-431-3p	0.02013327	2.05×10^{-4}	98.47	98.47	6.62	MI0001524
mmu-miR-127-3p	0.02179342	2.34×10^{-4}	8.23	8.23	3.04	MI0000154
mmu-miR-21a-5p	0.03119435	3.50×10^{-4}	1.89	1.89	0.92	MI0000569
mmu-miR-376b-3p	0.0328806	4.03×10^{-4}	124.07	124.07	6.96	MI0001162
mmu-miR-206-3p	0.04415612	6.31×10^{-4}	2.66	2.66	1.41	MI0000249
mmu-miR-370-3p	0.04681506	6.93×10^{-4}	7.72	7.72	2.95	MI0001165

Figure 3. *Cont.*

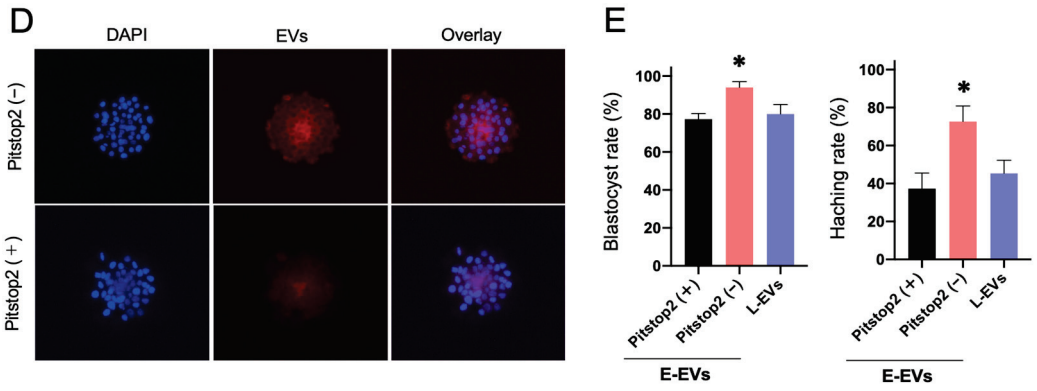


Figure 3. (**A**) The NanoSight instrument indicates the concentration or size of nanoparticles in the lower chamber. T-Control: no cells in the upper chamber; E-ASCs (adipose tissue-derived mesenchymal stem cells): the early passage of ASCs (P3: 3rd passage) seeded in the upper chamber; L-ASCs: late passage of ASCs (P20: 20th passage) seeded in the upper chamber. (**B**) Detection of exosomal markers CD63, CD81, and CD9 in exosomes isolated from the supernatant of E-ASCs or L-ASCs determined by Western blotting. * $p < 0.05$ vs. E-EVs. (**C**) Expression analysis of miRNAs in E-EVs and L-EVs determined by Next-Generation Sequencing. (**D**) Fluorescence microscopy showing the E-EVs taken up by the blastocyst. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). (**E**) Blastocyst formation and hatching rates of in vitro-produced mouse embryos with exosomes of E-ASCs (E-EVs) or exosomes of L-ASCs (L-EVs). E-ASC group with Pitstop2 (+) or without Pitstop2 (–). Data are presented as the mean ± standard error. * $p < 0.01$ vs. E-EVs with Pitstop2.

3.6. Effect of Exosomes on the Gene Expression of Blastocysts

We conducted a transcriptome analysis of the blastocysts grown from the 2-cell embryos in the KSOM (control [Ctrl] N = 8), the KSOM supplemented with E-ASCs-EVs (N = 8), and the KSOM supplemented with L-ASCs-EVs (N = 8). Based on our initial evaluation of the transcriptome data of 24 blastocysts by hierarchical clustering and multi-dimensional scaling (MDS) using EdgeR, we excluded two samples (Ctrl_14 and Ctrl_16) from the control group and one sample (E_ASC_7) as an outlier. The count data of the 21 samples were analyzed using the EdgeR 3.36.0. After excluding genes that were not expressed in any of the three sample groups, the count data for 13,292 genes was subjected to TMM normalization. The MDS analysis of the normalized data separated the 21 samples into three clusters: (Group 0 [G0], Group 1 [G1], and Group 2 [G2]) (Figure 4A). All six Ctrl samples were clustered together as Group0. Additionally, E_ASC and L_ASC samples were clustered into either Group 1 or Group 2. Group 1 contained one E_ASC and four L_ASC samples, whereas Group 2 contained six E_ASC and four L_ASC samples. Upon comparing Group 1 and Group 0, we identified 1318 up-regulated genes in Group 1. Those up-regulated genes in Group 1 were enriched with GO terms, such as "Nonsense Mediated Decay independent of the Exon Junction Complex", "Ribonucleoprotein complex biogenesis", "Detoxification of Reactive Oxygen Species", and "Mechanisms associated with pluripotency" (Figure 4B). When comparing Group 2 and Group 0, we identified 1965 up-regulated genes in Group 2. In a gene ontology (GO) analysis using Metascape, the up-regulated genes in Group 2 were found to be enriched with GO terms such as "Nonsense Mediated Decay independent of the Exon junction Complex", "Detoxification of Reactive Oxygen Species", "Mechanisms associated with pluripotency", and "In utero embryonic development." (Figure 4B). In the comparison of Group 2 and Group 1, we identified 1385 up-regulated genes in Group 2. Those up-regulated genes in Group 2 were enriched with GO terms such as "Actin cytoskeleton organization", "Embryonic organ development", "Embryonic placenta development", and "Mechanisms associated with pluripotency" (Figure 4B). EVs derived from the mesenchymal stem cells may induce the development of blastocyst, accompanied by a high expression of genes involved in the cell

cytoskeleton, pluripotency, and detoxication of reactive oxidative stress, and EVs derived from mesenchymal stem cells with distinct stemness may also generate the development of blastocysts as well as blastocyst outgrowth (Figure 4C).

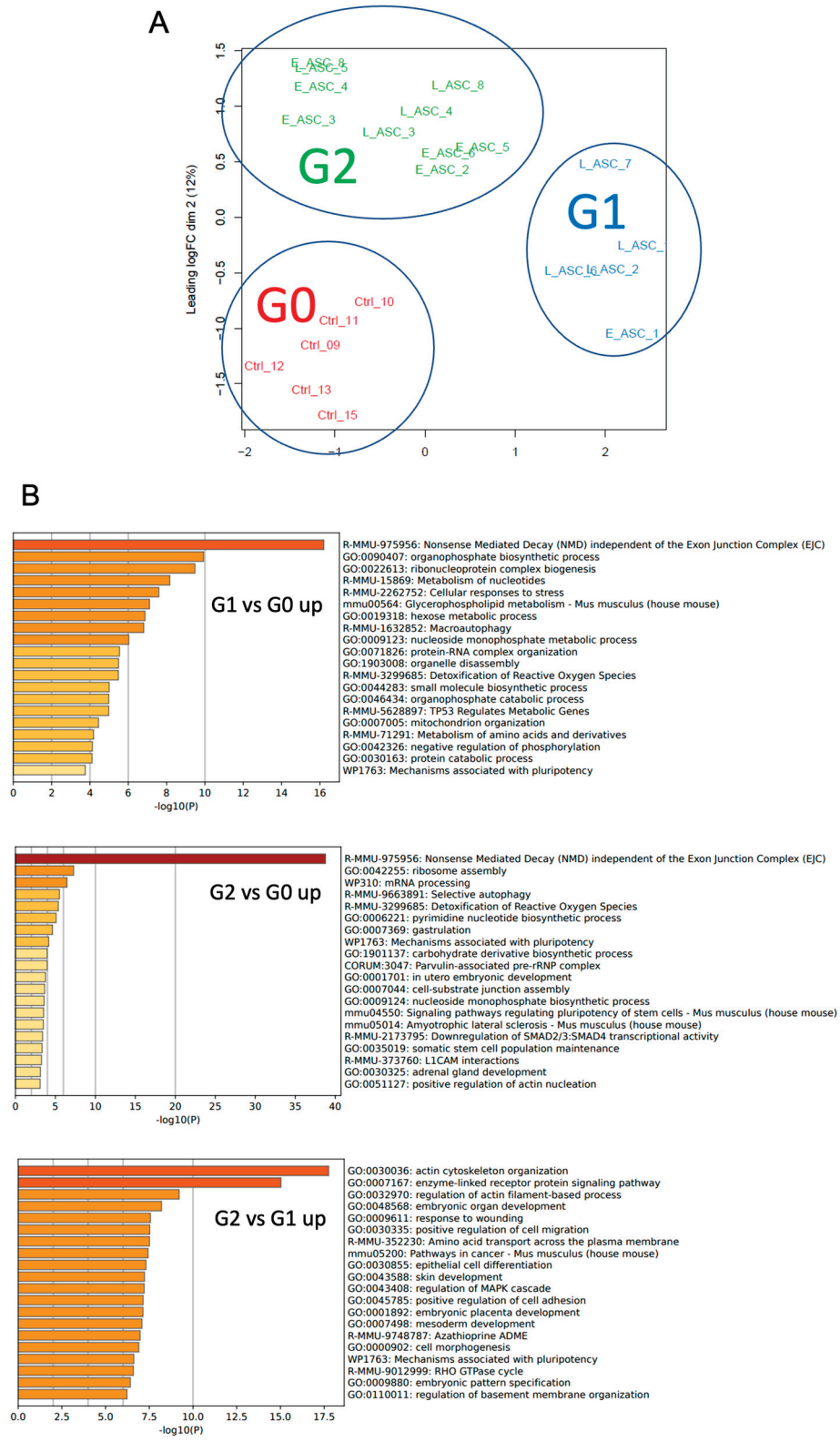


Figure 4. *Cont.*

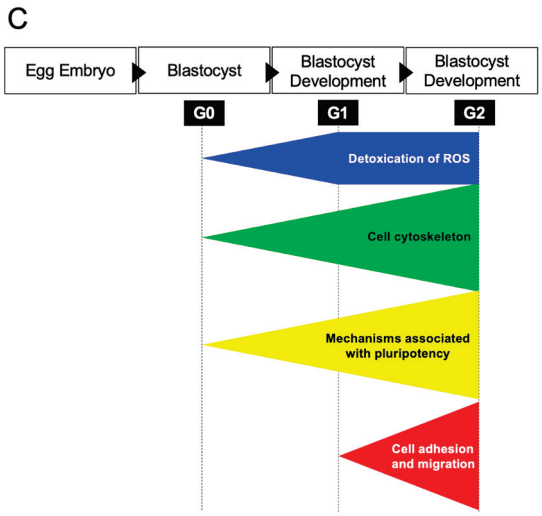


Figure 4. Effects of exosomes on the gene expression of blastocysts cultured in vitro. (**A**) Identification of three clusters based on transcriptome data. (**B**) Characterization of up-regulated genes affected by E-EVs and L-EVs. (**C**) Summary of the GO analysis among the three groups, G0, G1, and G2: the genes associated with the cell cytoskeleton, pluripotency, and detoxification of reactive oxidative stress were remarkably up-regulated in G2, compared with those in G0 and G1, and in G1, they also significantly increased, compared with those in G0. In addition, the genes associated with cell adhesin and migration in G2 indicated high expression compared with those in G1.

4. Discussion

In the Transwell culture, the fertilized eggs developed into blastocysts using the 3rd-passage stem cells, cultured in the upper layer, more efficiently than those using the 20th-passage stem cells and those without stem cells. In principle, it has been thought that ROS are endogenously generated in pre-implantation embryos as a by-product of ATP synthesis through oxidative phosphorylation and enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase and that physiological concentrations of ROS are indispensable for crucial embryonic processes, including pronuclear formation, first cleavage, and cellular proliferation [46,47]. Reportedly, high concentrations of reactive oxygen species induce oxidative stress damage to DNA, proteins, and lipids in cells [48–50]. This study observed an upregulation of genes associated with ROS detoxification through GO analysis conducted between the G0 and G1 blastocyst groups, as well as between the G0 and G2 blastocyst groups. As exosomes derived from mesenchymal stem cells can suppress oxidative stress with their antioxidant properties [51,52], the addition of exosomes derived from mesenchymal stem cells may progress the development of blastocysts in the Transwell culture. The developed blastocysts, which were incubated with stem cells, expressed genes associated with pluripotency and embryonic development better than those incubated without stem cells. EVs, including exosomes, which are produced by the 3rd-passage stem cells, participate in the development of blastocysts and possess the characteristics of specific miRNAs. Overall, Transwell culture systems using stem cells are recognized as promising tools for assisted reproductive technology.

In this study, the death of ASCs or fertilized eggs was evident in the culture media. The fertilized eggs were more developed into blastocysts because of the co-culture of ASCs and fertilized eggs in the mixed medium. Under culture conditions, the cell growth of ASCs was stopped. In the Transwell culture system, ASCs normally proliferated, leading to simultaneous significant development in fertilized eggs. In addition, fertilized eggs incubated with 3rd-passage stem cells showed the highest hatching frequency. According to these lines of evidence, blastocysts, which were efficiently developed from fertilized eggs in the Transwell culture system, could be technically feasible and clinically useful for uterine implantation.

The supplementation of IL-6, VEGF, and FGF2 has been documented to be advantageous in the differentiation of fertilized ova [53–55]. Additionally, VEGF and FGF are implicated in follicular maturation, as well as in the establishment and sustenance of the corpus luteum [56]. Moreover, IL6, VEGF, and FGF2 have all been demonstrated to facilitate implantation into the endometrium [57–59]. Hence, cytokines, such as IL6, VEGF, and FGF2 are frequently touted for their efficacy in enhancing fertility. Nevertheless, studies have reported on the association of elevated IL6 levels in tubal fluids with tubal factor infertility [60], casting uncertainty on its utility. In this study, the paracrine factors, including IL-6 and VEGFA, were not directly associated with blastocyst formation from fertilized eggs. The exosomes extracted from 3rd-passage stem cells significantly induced the formation of blastocysts from fertilized eggs better than those from 20th-passage stem cells. Principal component analysis revealed that exosomes from stem cells induced alterations in the genetic transcripts of all blastocysts. Furthermore, exosomes secreted from the same passage of stem cells did not always indicate constant quality. According to the GO analysis, the introduction of putative high-quality exosomes is involved in nonsense-mediated decay, ribosome assembly, and RNA processing. Furthermore, these genes have been associated with embryonic development [61–65]. In addition, genes associated with pluripotency, ROS detoxification, and embryonic development were highly expressed in blastocysts incubated with stem cells. Compared with the transcripts expressed in blastocysts incubated with stem cells, the genes associated with the actin cytoskeleton, embryonic development, and pluripotency were related to blastocysts introduced with putatively high-quality exosomes. According to these pieces of evidence, exosomes derived from stem cells may play pivotal roles in the maturation and development of fertilized eggs. In vivo, exosomes secreted from tubal epithelial cells are essential factors in the development of fertilized eggs [33]. In in vitro fertilization, the addition of exosomes may contribute to the appropriate embryonic development of fertilized eggs.

Exosomes extracted from 3rd-passage stem cells express specific miRNAs, such as MiR127, which is involved in mesendodermal differentiation during early embryogenesis [66] and placental development [67]. MiR127 may also be associated with ovarian function and testicular function [68–70]. Exosomes containing miR127 promoted the differentiation of mouse embryonic fibroblasts into β-like cells with a glucose metabolism function [71,72]. Exosomes harboring miR382 isolated from follicular fluids may play a role in regulating follicle maturation [73]. Additionally, miR382 may participate in the epithelial–mesenchymal transition [74]. It has been reported that miR-181a-2-3p and miR-200c-3p exhibit antioxidant properties [75,76]. As fertilized eggs cultured in vitro are subjected to oxidative stress induced by ROS, the antioxidant properties of these miRNAs may exert a beneficial influence on embryonic development. In this study, the examination of gene expression in exosomes extracted from 3rd-passage stem cells revealed an upregulation in the expression of these miRNAs, which could reinforce this phenomenon. Furthermore, there are many reports concerning miR141-3p, miR676-3p, miR429-3p, miR379-5p, and miR154-5p, which have been associated with the pathogenesis of various cancers and other disorders [77–81]. Therefore, these miRNAs may play promising roles in reproduction and embryonic development.

IL-6 promotes the expression of miRNAs in exosomes [82], which can induce the secretion of IL-6 and IL-8 [83]. Silencing IL-6 significantly inhibits the proliferation of human mesenchymal stem cells. Furthermore, increasing extracellular IL-6 levels cannot restore the proliferative impairment observed in IL-6-silenced human mesenchymal stem cells [84]. In the present study, IL-6 had no effect on blastocyst formation from fertilized eggs. IL-6, VEGFA, and exosomes were better expressed in 3rd-passage stem cells than in 20th-passage stem cells. Based on these findings, it is plausible that IL-6 participates in stemness retention. Unlike VEGF, exosomes promote the crosstalk between inflammation and angiogenesis [85]. However, there are only a few reports suggesting a possible role of VEGF in stimulating exosome production by stem cells. Consequently, IL-6 and VEGF may promote tissue regeneration in the presence of exosomes.

This study demonstrates that exosomes released by ASCs could enhance the differentiation capacity of fertilized ova and characterizes the specific miRNAs that are abundantly present in these exosomes. This study had some limitations. Firstly, the investigation lacked a comprehensive determination of the optimal exosome dosage or the efficacy of individual miRNAs in facilitating ova differentiation. The findings regarding alterations in gene expression in fertilized ova indicate a plausible scenario wherein diverse miRNAs may interact intricately across multiple pathways, warranting elucidation in future research endeavors. Furthermore, the culture technique utilized in this study, employing the Transwell system, is structurally distinct from the conventional culture method currently used in human ARTs. Therefore, when translating the results of this research to human ART techniques, it is challenging to apply the Transwell system directly, necessitating the development of methodologies compatible with actual clinical practice. However, as platelet-rich plasma reportedly plays a role in oocyte maturation and fertilized egg differentiation, the extraction and purification of exosomes using autologous mesenchymal stem cells may render this technology viable for clinical application [86]. Mesenchymal stem cell-derived exosome therapy may have limited clinical application owing to the ethical issues involved. Some countries have approved exosome therapy as long as it is derived from the individual, while others have approved it for exosome therapy derived from others; thus, the views are not uniform. We hope that our research will provide an opportunity to resolve ethical issues.

5. Conclusions

In conclusion, in assisted reproductive technologies, the pregnancy rate has been investigated using in vitro-produced embryos. In this study, exosomes secreted from stem cells helped fertilized eggs develop into blastocysts with high hatching potential. Blastocysts express many transcripts that are required for excellent embryonic development. Exosomes from tubal epithelial cells can be inserted into blastocysts during normal pregnancy. Closer to the living body, therefore, the Transwell culture system should be recognized as a beneficial and favorable tool in in vitro fertilization, which may help pregnancy rates in countries with low birth rates. Future studies examining the optimal exosome dosage or the efficacy of individual miRNAs in facilitating ova differentiation may help validate our findings.

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Cord Blood Adductomics Reveals Oxidative Stress Exposure Pathways of Bronchopulmonary Dysplasia

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Abstract: Fetal and neonatal exposures to perinatal oxidative stress (OS) are key mediators of bronchopulmonary dysplasia (BPD). To characterize these exposures, adductomics is an exposure science approach that captures electrophilic addition products (adducts) in blood protein. Adducts are bound to the nucleophilic cysteine loci of human serum albumin (HSA), which has a prolonged half-life. We conducted targeted and untargeted adductomics to test the hypothesis that adducts of OS vary with BPD. We studied 205 preterm infants ($\leq$28 weeks) and 51 full-term infants from an ongoing birth cohort. Infant plasma was collected at birth (cord blood), 1-week, 1-month, and 36-weeks postmenstrual age. HSA was isolated from plasma, trypsin digested, and analyzed using high-performance liquid chromatography–mass spectrometry to quantify previously annotated (known) and unknown adducts. We identified 105 adducts in cord and postnatal blood. A total of 51 known adducts (small thiols, direct oxidation products, and reactive aldehydes) were increased with BPD. Postnatally, serial concentrations of several known OS adducts correlated directly with supplemental oxygen exposure. The application of large-scale adductomics elucidated OS-mediated pathways of BPD. This is the first study to investigate the "neonatal–perinatal exposome" and to identify oxidative stress-related exposure biomarkers that may inform antioxidant strategies to protect the health of future generations of infants.

Keywords: addition products; neonate; pre-eclampsia; chorioamnionitis; intrauterine growth restriction

1. Introduction

Bronchopulmonary dysplasia (BPD) remains the most common chronic lung disease of infancy, affecting over half of extremely preterm (EPT) infants worldwide [1]. Severe BPD is characterized by chronic supplemental oxygen exposure, mechanical ventilation dependence, prolonged and recurrent hospitalizations within the first year of life, and long-term cardiopulmonary, metabolic, and neurodevelopmental problems [2–6]. Putative early-life exposures include pre-eclampsia and uteroplacental insufficiency during pregnancy, and hyperoxia in the early postnatal period [7]. Other maternal and neonatal exposures contributing to BPD have yet to be identified, but an important scientific premise is that these exposures collectively contribute to a common putative pathway of elevated oxidative stress (OS) [8].

Reactive oxygen and nitrogen species (ROS/RNS) are central to BPD pathophysiology as they directly damage DNA and the proteins necessary for lung growth and repair [9–11].

These electrophiles enter the blood from absorption in the lungs or via metabolism in the liver and other tissues, from oxidation of lipids and other molecules, and from inflammation associated with co-existing disease states. Once entering the blood, electrophiles react with available proteins to form addition products, or "adducts" [12]. When bound to stable proteins such as human serum albumin (HSA), these adducts become significantly more stable (28 days in circulation) than the scavenged reactive electrophiles [12], and thus may serve as more reliable biomarkers of exposure in chronic disease states, such as BPD.

The HSA-Cys^{34} residue (Figure 1) is a sentinel nucleophilic hotspot for protein adducts that accounts for 80% of the antioxidant capacity of serum/plasma [13]. HSA-Cys^{34} adducts potentially play a pivotal role in understanding human disease arising from exposures to electrophilic species [12,14]. Until recently, studies addressing HSA-Cys^{34} adducts have been mainly focused on untargeted assays. While required for discovering unknown HSA-Cys^{34} adducts, untargeted approaches typically have lower analytical sensitivity than targeted assays. Our recently developed targeted assays can simultaneously quantify large panels of HSA-Cys^{34} adducts with higher detection rates than untargeted assays [12,14,15]. The application of untargeted adductomics in an EPT infant population would provide new opportunities to investigate the "neonatal exposome" as it relates to BPD. Once identified, oxidative stress-related exposure pathways and novel biomarkers could be further characterized that predict disease, track meaningful responses to perinatal and NICU exposures, and inform the development of therapies to prevent BPD.

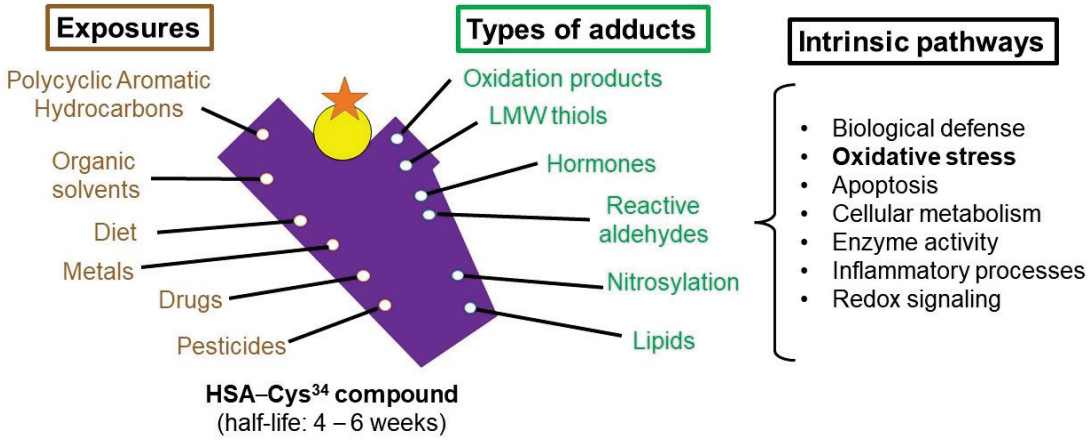


Figure 1. The human serum albumin–cysteine^{34} compound captures addition products (adducts) from variable exposures affecting downstream biological pathways. The purple shape represents the HSA protein. The yellow circle represents the Cys^{34} residue. The red star represents an adduct within the HSA-Cys^{34} compound. The green and brown terms indicate environmental exposures, which are mostly reactive and potentially toxic electrophiles. The black terms indicate potential adverse biological mechanisms, which can be induced by toxic electrophiles.

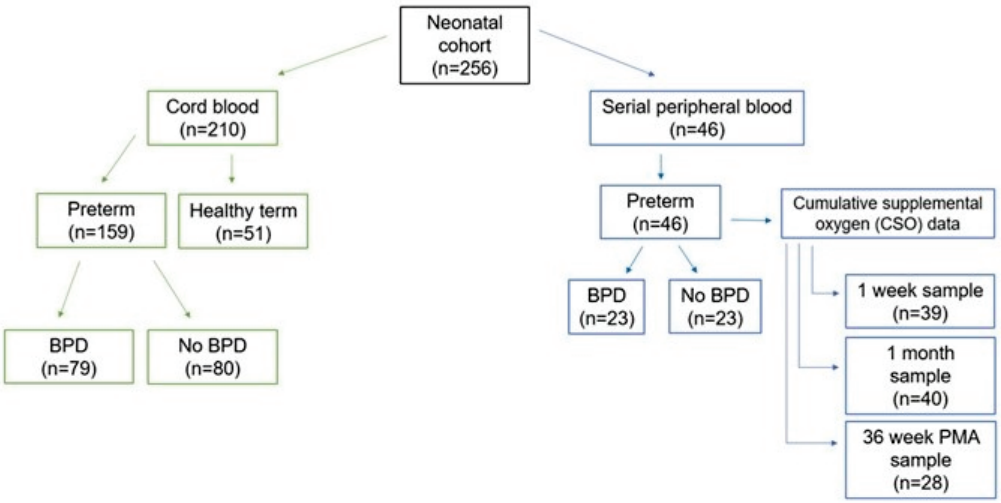
We employed novel exposomic approaches in a single-center study of EPT and healthy full-term (FT) infants. Our primary objective was to identify cord blood HSA-Cys^{34} adducts that are associated with BPD. In postnatal blood from EPT infants, we sought to further identify HSA-Cys^{34} adducts that correlate with cumulative supplemental oxygen (CSO) exposure in the postnatal period. We hypothesized that the adductomics profiles would elucidate perinatal and postnatal mechanisms of OS-mediated BPD.

2. Materials and Methods

2.1. Study Design and Patient Sample

Patients were drawn from an ongoing birth cohort study conducted at Prentice Women's Hospital (Chicago, IL, USA) from 2008 to 2021. As shown in Figure 2, there were two arms of the study: (1) The cord blood arm comprised 210 births (159 EPT and 51 FT) with corresponding archived cord blood plasma samples which were drawn from

the larger birth cohort. A total of 79 BPD and 80 non-BPD infants were included in the final analysis, in addition to 51 healthy FT infants (born at ≥37 completed weeks without newborn complications) who were enrolled over the same time period. Informed consent was obtained from mothers prior to participation. The details of enrollment and sample/data collection, processing, and archive in the parent cohort have been published previously and are described elsewhere [16–18]. (2) The peripheral blood arm included 46 EPT infants who were followed prospectively from 2020 to 2021 with scavenged postnatal blood samples recovered from scheduled blood draws performed at 1-week, 1-month, and 36-weeks postmenstrual age (PMA). The samples were linked to clinical data throughout each patient's NICU hospitalization, including respiratory data and cumulative supplemental oxygen exposure (see below). Informed consent was waived under a scavenged biospecimen protocol that was linked to coded data that were deidentified upon NICU discharge. There was no patient overlap between cord blood and peripheral blood arms.



	Cord Blood		Peripheral Blood	
	No BPD (n=80)	**BPD (n=79)**	**No BPD (n=23)**	**BPD (n=23)**
Gestational age (w)	27.5 ± 1.2	26.2 ± 1.6*	28.3 ± 0.5	25.9 ± 1.7*
Birth weight (g)	1080 ± 204	870 ± 200*	1190 ± 169	800 ± 204*
Male sex, n (%)	39 (48.8)	40 (50.6)	19 (82.6)	10 (43.5)
SGA	1 (1.3)	6 (7.6)	0 (0)	3 (13.0)

*$P<0.001$; SGA=small-for-gestational age defined as <10th percentile birth weight-for-GA

Figure 2. A breakdown of the infant cohort by cord blood and peripheral blood groups. Each serial peripheral blood sample was linked to respiratory data that included a calculation of cumulative supplemental oxygen (CSO). None of the infants from the cord blood arm (left) overlapped with the serial peripheral blood arm (right). As shown in the table, differences in baseline characteristics were similar between the two arms.

2.2. Clinical Data Collection

Maternal and infant information was extracted from electronic medical records using standardized protocols and automated programs in consultation with Northwestern's Enterprise Data Warehouse (EDW). This included detailed information on antepartum, intrapartum, and pregnancy outcomes, and serial data on infant hospitalization course (see below) for each mother–infant dyad enrolled in the birth cohort. Maternal pre-eclampsia and other hypertensive disorders of pregnancy (eclampsia, and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome) were defined according to American College of Obstetrics and Gynecology (ACOG) criteria. Chorioamnionitis was defined as

having either clinical evidence according to ACOG criteria or histologic evidence of acute inflammation by placental pathology exam [19–22]. Small for gestational age (SGA) was defined as birth weight < 10th percentile for GA based upon Fenton growth curves for premature infants [23].

Detailed NICU data were collected on all EPT infants. This included serial information related to the primary outcome of BPD, which was defined using the modified NIH criteria [24] and included Grade I, II, III, or III(A) as determined at 36 weeks PMA. Neonatal complications of necrotizing enterocolitis (NEC), intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), and BPD-associated pulmonary hypertension (BPD-PH) were defined using our standardized protocols for the cohort, as previously published [16,18,25]. Respiratory support modes and FiO_2 values at 8 h intervals (07:00, 15:00, and 23:00) were captured using automated electronic medical record algorithms. The calculation of cumulative supplemental oxygen (CSO) was adapted from a published algorithm as the sum of average daily supplemental oxygen (FiO_2–0.21) over the time interval leading up to blood collection at 1-week, 1-month, and 36-weeks PMA [26]. CSO values at each timepoint were divided into quartiles and categorized for each patient as low (<25th percentile of the distribution), mild (25–50th percentile), moderate (51–75th percentile), and high CSO (>75th percentile of the distribution).

2.3. Cord Blood and Postnatal Peripheral Blood Collection

Umbilical venous cord blood was collected at birth into ethylenediaminetetraacetic acid (EDTA) tubes, aliquoted, and separated for plasma by tabletop centrifugation at 3000 rpm at 4 °C for 10 min. The plasma was archived at −80 °C until assay. Left-over peripheral blood plasma samples were retrieved from the Northwestern Memorial Hospital chemistry lab within 24 h after routine patient blood draws at 1-week, 1-month, and 36-weeks PMA, and stored at −80 °C until assay.

2.4. Plasma Adductomics

Chemical Reagents. EDTA (BioUltra, anhydrous, ≥99%), triethylammonium bicarbonate buffer (TEAB) (1 M, pH 8.5), sodium hydroxide (BioXtra, ≥98%), HSA (lyophilized powder, ≥96%, agarose gel electrophoresis), and trypsin (from porcine pancreas, lyophilized powder, 1000–2000 BAEE units/mg solid) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (Optima LC/MS grade), acetonitrile (Optima LC/MS grade), and methanol (HPLC grade grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). T3 peptide with the sequence ALVLIAFAQYLQQCPFEDHVK (>95%, LCMS Pure, 2433 g/mol) was custom synthesized by the Peptide Core in Northwestern University (Chicago, IL, USA). Here, it is important to note that the T3 peptide contains Cys^{34}, which can promote the formation of protein adducts. All of the stock solutions and samples were prepared using deionized (DI) water (resistivity > 18.2 mΩ-cm) prepared with a PureLab purification system (ELGA LabWater, Woodridge, IL, USA).

Isolation of HSA from Plasma. A total of 60 µL of a mixture containing 50% methanol and 50% DI water was added to 5 µL aliquots of plasma to precipitate unwanted proteins. The mixture was mechanically shaken for 30 min followed by centrifugation for 15 min (14,000 rpm, 4 °C). Thirty microliters of the resulting supernatant was then transferred to protein digestion tubes (MT-96, Pressure Biosciences Inc., South Easton, MA, USA) and diluted with 120 µL of digestion buffer (50 mM triethylammonium bicarbonate and 1 mM ethylenediaminetetraacetic, pH 8.0). A total of 3 µL of 10 µg/µL trypsin (1:30 *m*/*m* trypsin/HSA) was added and protein digestion was performed using a Pressure Biosciences™ Barozyme HT48 system. After digestion, 3 µL of 10% (*v*/*v*) formic acid in DI water was added to each sample and briefly centrifuged at 14,000 rpm. A 40 µL aliquot was then mixed with 80 µL of 2% acetonitrile and 0.2% formic acid (*v*/*v*) in DI water and centrifuged at 14,000 rpm for 15 min. One-hundred microliter aliquots of supernatant were then transferred to borosilicate silanized glass vials (Microsolv Technology Corporation, Leland, NC, USA) prior to analysis.

Untargeted Adductomics. Untargeted adductomics were initially performed on 16 cord blood plasma samples across a gestational age spectrum from 32 to 40 weeks. This served to identify all known adducts with corresponding structures as well as to detect unknown adducts with new organic structures for analysis. Once all adducts were validated, targeted adductomics results could be compared to validated known and unknown adducts from untargeted adductomics.

The discovery experiments were performed using an Orbitrap LTQ Velos Elite Orbitrap (Thermo Fisher, San Jose, CA, USA) equipped with a Dionex Ultimate 3000 RSLC nano system. A total of 5 µL of HSA protein digest was loaded onto a trap column (5 mm × 0.2 mm i.d., Thermo PepSwift™ Monolithic capillary column) with a flow rate of 5 µL/min for 5 min. A Thermo PepSwift™ Monolithic capillary analytical column (250 mm × 0.1 mm i.d.) with a custom nanospray source and a stainless-steel emitter was used for peptide separation (outer diameter 150 um; inter diameter 30 um). Solvent A contained 0.1% formic acid in LC/MS grade water and solvent B contained 0.1% formic acid in LC/MS grade acetonitrile. A 750 nl/min flow rate was used with the following gradient conditions: 0–7 min at 2% solvent B; 7–40 min, linear gradient from 2–40% B; 40–45 min, 98% B; 45–50 min, 2% B, with a total run time of 50 min. High-resolution data files were manually inspected using the Xcalibur™ Software (Version 4.1.50) to identify new adduct features. Total ion chromatograms (TICs) were examined stepwise in 0.088 min increments across retention times ranging from 20 to 60 min. Three *b* ions—b_3^+ (m/z = 284.42), b_4^+ (m/z = 397.63), and b_5^+ (m/z = 510.84)—were used to identify T3 peptides containing Cys^{34} adducts. A second series of three y^+-series ions, including y_{15}^+ (m/z = 973.92 + α), y_{16}^+ (m/z = 1009.47 + α), and y_{17}^+ (m/z = 1066.07 + α), were then used to determine the added mass corresponding to new adduct features. Newly identified adduct features were then added to our targeted adductomics panel in subsequent experiments for adduct quantitation.

Targeted Adductomics. Targeted adductomics was performed using an Agilent 6490 QqQ with an iFunnel electrospray source coupled to an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA). An Agilent Poroshell 120 EC-C^{18} LC column was used for peptide separation (50 × 3 mm, 2.7 µm). Ten microliters of HSA digest was injected per sample and multiple reaction monitoring (MRM) was used to monitor precursor ions corresponding to triply charged T3 adducted peptides (y_{15}^{2+}, y_{16}^{2+}, and y_{17}^{2+}). Solvent A consisted of 0.1% formic acid in LC/MS grade water, and solvent B consisted of LC/MS grade acetonitrile. Adduct quantification and data processing are described in detail in previously published studies [27–29]. Raw data files were imported to Skyline (Ver. 21.1) with a complete set of transitions for each run [15]. Peaks were manually selected using 3 transitions based on nominal mass and retention time (y_{15}^{2+}, y_{16}^{2+}, and y_{17}^{2+}). Quantitation was based on the summed peak areas of the three transitions, normalized to a housekeeping peptide (HKP) [14]. The HKP is a doubly charged precursor ion adjacent in sequence to the T3 tryptic peptide (^{41}LVNEVTEFAK^{50}), which is used to normalize adducts concentrations to the amount of HSA in each sample [15]. All statistical analyses were performed using adduct peak areas/HKP peak area (PAR). PAR values were also converted to pmol of adduct/mg of HSA using two external calibration curves to estimate adduct concentrations (see Supplemental Figure S1).

2.5. Statistical Analysis

Continuous data were analyzed using parametric or non-parametric tests, where appropriate. Categorical data were compared using Fisher's exact or Chi-square tests. All adduct concentrations were non-normally distributed. To take this into account, adduct concentrations were scaled by log-transformation and normalized with Trimmed Means of M-Values (TMM) normalization to account for concentration variations between samples (TMM normalization excludes a percentage of upper and lower values, takes a trimmed mean, and generates scale factors to differentiate between samples). Mean adduct concentrations were analyzed with R software (Ver. 4.0.3) using the Bioconductor packages edgeR

and limma [30]. Linear regression models were used to identify differentially expressed adducts by relevant clinical metadata variables between BPD, non-BPD, and healthy full-term (FT) infant controls. Statistical significance was based on a Bonferroni-adjusted p-value < 0.05 (P_{adj}) and significance thresholds for adductomics results are reported using $P_{adj} < 0.05$ and $P_{adj} < 0.001$.

3. Results

3.1. Characteristics of the Patient Cohort

Figure 2 illustrates the distribution of all 256 infants included in this study. Table 1 shows the baseline demographics and clinical characteristics of the patient sample. Of the 205 EPT infants, 102 infants had BPD and 103 did not, according to the study definition for BPD [24]. The three groups (FT vs. non-BPD vs. BPD) differed by maternal age and antenatal diagnosis of intrauterine growth restriction (IUGR), with a higher rate of IUGR in the BPD group ($p < 0.001$). Compared to non-BPD preterm infants, BPD infants had a lower gestational age (GA), a lower birth weight (BW), and lower APGAR scores, and were more likely to be SGA. Co-morbidities of NEC, IVH, ROP, and BPD-PH were increased with BPD.

Table 1. Baseline demographics and clinical characteristics of the patient sample.

	Full Term (n = 51)	**No BPD (n = 103)**	**BPD (n = 102)**	***p* ***
MATERNAL				
Maternal age, years, (mean ± SD)	34.6 ± 4.1	30.5 ± 5.6	32.3 ± 5.6	0.02
Maternal race, n (%)				
White	40 (78.4)	41 (39.8)	42 (41.2)	0.1
Black/African American	3 (5.9)	30 (29.1)	24 (23.5)	
Asian	1 (2.0)	5 (4.9)	7 (6.9)	
American Indian/Alaska Native	0 (0)	2 (1.9)	2 (2.0)	
Other	5 (9.8)	19 (18.4)	16 (15.7)	
Declined/Unknown	2 (3.9)	6 (5.8)	11 (10.8)	
Ethnicity, n (%)				
Hispanic or Latino	10 (19.6)	25 (24.3)	18 (17.6)	0.47
Not Hispanic or Latino	41 (80.4)	73 (70.9)	75 (73.5)	
Declined/Unknown	0 (0)	5 (4.9)	9 (8.8)	
Smoking status, n (%)				
Current smoker	2 (3.9)	4 (3.9)	0 (0)	0.05
Former smoker	4 (7.8)	14 (13.6)	8 (7.8)	
Never smoker	42 (82.4)	43 (41.7)	38 (37.3)	
Unknown	3 (5.9)	42 (40.8)	56 (54.9)	
Rupture of membranes, n (%)				
Artificial	42 (82.4)	50 (48.5)	49 (48)	1
Spontaneous	9 (17.6)	52 (50.5)	53 (52)	
Delivery method, n (%)				
Cesarean	7 (13.7)	58 (56.3)	68 (66.7)	0.17
Vaginal	44 (86.3)	45 (43.7)	34 (33.3)	
Twin delivery, n (%)	2 (3.9)	33 (32)	30 (29.4)	0.80

Table 1. *Cont.*

	Full Term (n = 51)	No BPD (n = 103)	BPD (n = 102)	*p* *
Antenatal steroids, n (%)				
None	51 (100)	6 (5.8)	6 (5.9)	0.32
Complete	0 (0)	83 (80.6)	74 (72.5)	
Incomplete	0 (0)	14 (13.6)	22 (21.6)	
Preterm labor	0 (0)	75 (72.8)	64 (62.7)	0.16
Preterm premature rupture of membranes	0 (0)	51 (49.5)	48 (47.1)	0.83
Prolonged rupture of membranes	3 (5.9)	31 (30.1)	31 (30.4)	1
Pre-eclampsia/HELLP	1 (2)	17 (16.5)	27 (26.5)	0.14
Antenatal IUGR	0 (0)	2 (1.9)	16 (15.7)	0.001
Non-reassuring fetal heart tones	3 (5.9)	14 (13.6)	19 (18.6)	0.43
Chorioamnionitis	49 (96.1)	73 (70.9)	68 (66.7)	0.62
Diabetes, n (%)				
None	49 (96.1)	98 (95.1)	94 (92.2)	0.67
Gestational	2 (3.9)	4 (3.9)	6 (5.9)	
Insulin-dependent	0 (0)	1 (1)	2 (2)	
INFANT				
Gestational age, weeks, (mean ± SD)	39.5 ± 1.0	27.7 ± 1.2	26.1 ± 1.6	<0.001
Gestational age category, n (%)				
22–24 weeks	-	1 (1.0)	25 (24.5)	<0.001
25–26 weeks	-	25 (24.3)	39 (38.2)	
27–29 weeks	-	77 (74.8)	38 (37.3)	
Infant sex, n (%)				
Female	25 (49)	45 (43.7)	52 (51)	0.37
Male	26 (51)	58 (56.3)	50 (49)	
Birth weight (g), (mean ± SD)	3430 ± 452	1110 ± 201	854 ± 202	<0.001
Birth-weight-for-GA percentile (%)	52.8 ± 26.3	64.3 ± 21.2	56.3 ± 28.4	0.02
Small-for-gestational-age status, n (%)	2 (3.9)	1 (1)	9 (8.8)	0.02
APGAR score, median [min, max] 1-min 5-min	 8 [2, 9] 9 [8, 9]	 6 [0, 9] 8 [1, 9]	 4 [0, 8] 7 [1, 9]	<0.001
BPD grade, n (%)				
Grade I	-	0 (0)	33 (32.4)	--
Grade II	-	0 (0)	26 (25.5)	
Grade III/IIIA	-	0 (0)	43 (42.2)	
Other neonatal complications, n (%)				
Necrotizing enterocolitis (any)	0 (0)	6 (5.8)	24 (23.5)	<0.001
Intraventricular hemorrhage (any)	0 (0)	24 (23.3)	42 (41.2)	<0.001
Retinopathy of prematurity (stage 2+)	0 (0)	9 (8.7)	32 (31.4)	<0.001
Pulmonary hypertension @ 36 weeks	0 (0)	8 (7.8)	28 (27.5)	<0.001

* *p*-value calculated comparing BPD versus non-BPD groups, using Chi-square or Fisher's exact tests for categorical data, and parametric/non-parametric tests where appropriate.

3.2. Cord Blood Adductomics

Among the 210 infants, a total of 105 adducts were detected. Of these, 49 were unknown adducts and 56 were previously annotated (known) adducts. All adducts identified (assigned A001 to A105 for this study) and putative nomenclature and characteristics are shown in Supplemental Table S1. Samples were analyzed in duplicate and averaged across runs. The median of normalized concentrations for each adduct according to the study groups are shown in Supplemental Table S2. Previously annotated adducts were also assigned to functional categories based upon their chemical class [14,31–36], with relative abundance shown in Figure 3.

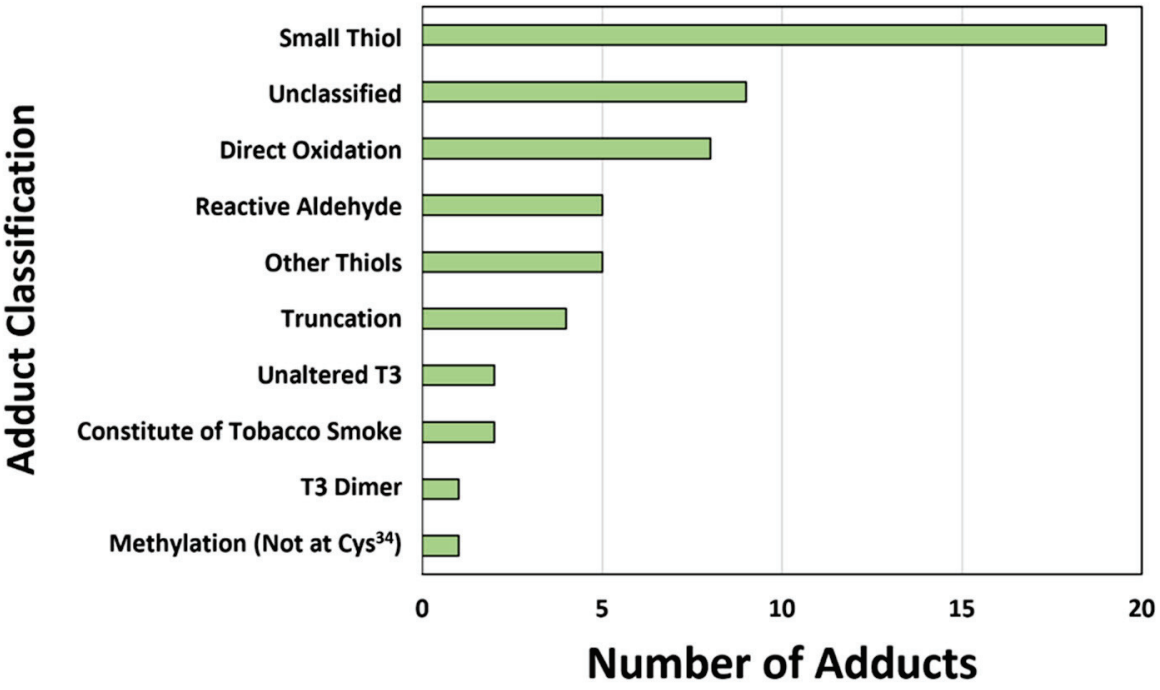


Figure 3. Previously annotated adduct classifications. Adducts were classified based on exogenous sources (e.g., tobacco smoke) and underlying endogenous processes (e.g., oxidative stress). Small thiols are Cys^{34} disulfides formed with small thiol molecules related to antioxidant capacity. Direct oxidation products are the addition of 1–3 oxygen molecules formed by reactions with Cys^{34} and ROS. Reactive aldehydes are reaction products formed through lipid peroxidation. Truncation products are reactions that result in the loss of mass at Cys^{34}. Methylation (not at Cys^{34}) refers to methyl groups added to the T3 peptide on amino acids other than Cys^{34}. Unaltered T3 are T3 peptides in their reduced form, with no adducts on Cys^{34}. Other disulfides are small thiols related to the microbiome and other sources not involved in antioxidant capacity. The constituents of tobacco smoke are exogenous adducts associated with tobacco smoke exposure. T3 dimers are disulfides formed between two T3 peptides.

3.2.1. Known OS Adducts in Cord Blood Were Associated with Extreme Prematurity

We compared the 56 previously annotated (known) adduct concentrations according to EPT versus FT status (Figure 4A). There were 50 known adducts that were increased with EPT (Figure 4B). In addition, unmodified T3 (A006) was elevated with EPT, with 1.2-fold increase when compared to the FT group ($P_{adj} < 0.001$).

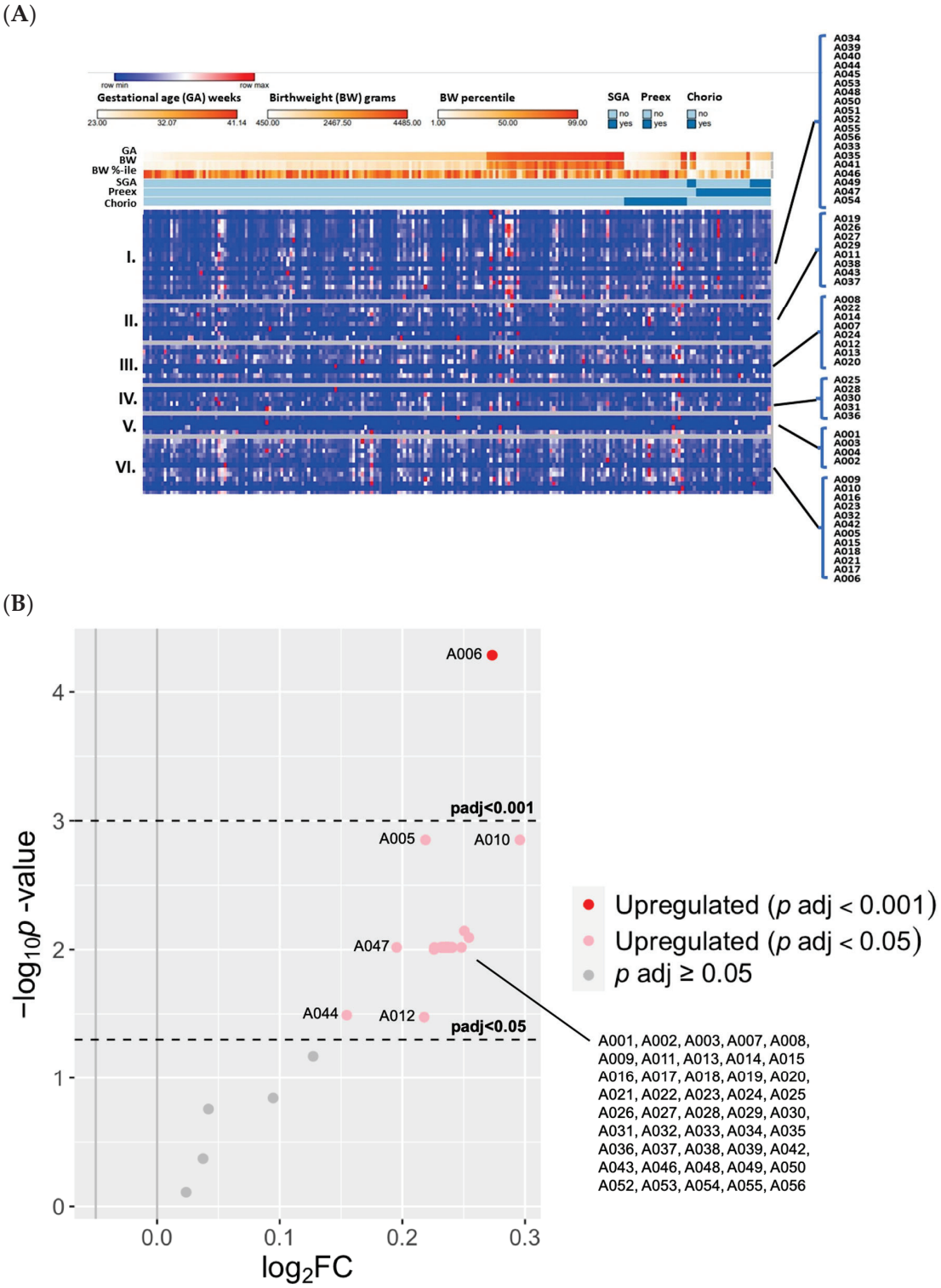


Figure 4. The differences in previously annotated (known) adduct concentrations according to gestational age and covariates of preterm birth. (**A**) A heatmap of relative concentrations of the 56 known adducts (listed on right), and grouped by (I) small thiols, (II) unclassified, (III) direct oxidation, (IV) reactive aldehydes, (V) truncation, and (VI) others. Heatmaps were generated using Morpheus https://software.broadinstitute.org/morpheus (accessed on 13 September 2023). (**B**) A volcano plot of upregulated known adducts with EPT versus FT infants. Significantly different adduct levels were filtered by a log fold-change of 2 and adjusted p values of $P_{adj} < 0.05$ and $P_{adj} < 0.001$.

3.2.2. Known OS Adducts Were Associated with Peripartum Exposures of Prematurity

To further explore the role of in utero exposures that might contribute to oxidative stress, we conducted subsequent analyses of the EPT group with respect to major complications preceding preterm birth. We focused specifically on clinical evidence of maternal pre-eclampsia and chorioamnionitis. Of the 159 EPT infants, 24 had exposure to maternal pre-eclampsia. Any preterm infant with chorioamnionitis was excluded and we confirmed that there were no infants with both pre-eclampsia and chorioamnionitis. With pre-eclampsia alone, we found a 1.5-fold increase in Cys^{34} sulfinic acid (A012; $P_{adj} = 0.001$). In addition, two adducts had decreased levels with pre-eclampsia (at least 1.1 FC; A005 and A006; $P_{adj} < 0.05$). In EPT infants exposed to maternal chorioamnionitis (N = 19), we found decreased levels of multiple small thiols (S-Addition of CysGly (A051), S-Cys (A040), S-Addition of hCys (A044), S-Additions of Cys (NH2→OH; A041), Na adduct of S-Cys (A047), S-Addition of Cys, methylation (A045), and S-Sodiation (A010; at least 1.1 FC; $P_{adj} < 0.05$).

3.2.3. Known OS Adducts Were Associated with BPD and BPD-Associated Pulmonary Hypertension

A heatmap of relative concentrations among the 159 EPT infants, according to major categories of known adducts, is shown in Figure 5A. The same known adducts found in the EPT versus FT analysis were associated with BPD versus non-BPD (≥1.1 FC; $P_{adj} < 0.05$), in addition to Cys^{34}→Oxoalanine (A004), with a 1.4-fold increase with BPD ($P_{adj} < 0.001$) (Figure 5B). We also performed a subgroup analysis comparing levels from BPD infants with PH at 36 weeks (BPD-PH; N = 25) versus BPD without PH (BPD only; N = 54). Fifty-four adducts were decreased with BPD-PH versus BPD only ($P_{adj} < 0.05$) (Figure 5C). The adduct most notably decreased with BPD-PH was a dehydrated form of Cys^{34} sulfinic acid plus methylation (not on Cys^{34}) (A012), with a 1.7-fold decrease ($P_{adj} < 0.001$). As shown in Figure 5A, infants with late PH (≥36 weeks PMA) with or without BPD tended to have lower adduct concentrations, except for one patient with BPD-PH (born at 27 weeks) who was exposed to chorioamnionitis in utero and died at 36-weeks PMA.

(**A**)

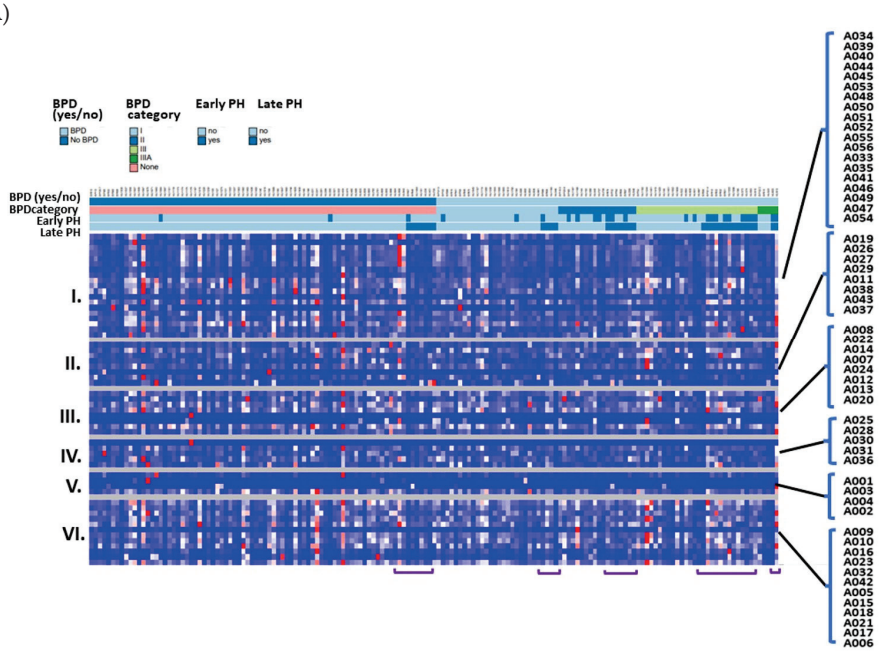


Figure 5. *Cont.*

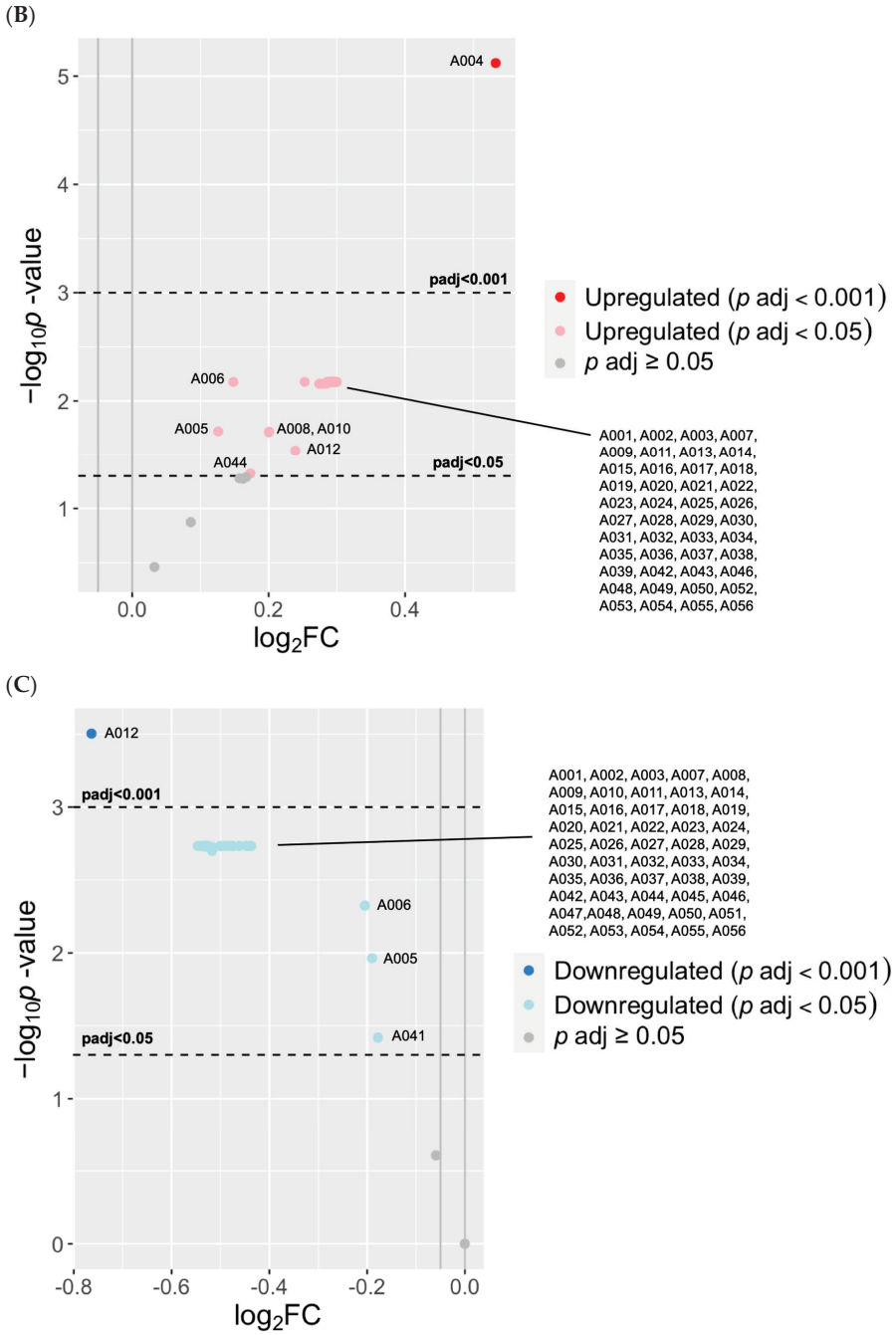


Figure 5. The differences in known adduct concentrations according to BPD and pulmonary hypertension. (**A**) A heatmap showing the relative concentrations according to BPD status and modified NIH categories (I-IIIA), and the presence of PH at ≤1-month (early PH) and ≥36-weeks PMA (late PH). Overall, the BPD group (right) appeared to have lower concentrations across all adduct classes. Infants with late PH, with or without BPD, tended to have lower adduct concentrations (purple brackets). One exception was a BPD-PH infant with relatively high adduct levels (far right) who presented with chorioamnionitis and died of bacterial sepsis. (**B**) A volcano plot comparing BPD and non-BPD controls. Significantly different adduct levels were filtered by a log fold-change of 2 and adjusted *p* values ($P_{adj} < 0.05$ and $P_{adj} < 0.001$). Most notable was Cys^{34}→Oxoalanine (A004), a truncation adduct increased with BPD ($P_{adj} < 0.001$). (**C**) A volcano plot comparing the known adducts according to BPD-PH versus BPD without PH (BPD only) infants. Most notable was a dehydrated form of Cys^{34} sulfinic acid plus methylation (A012), a direct oxidation product that decreased with BPD-PH ($P_{adj} < 0.001$).

3.3. Peripheral Blood (Postnatal) Adductomics

Characteristics of the 46 EPT infants in the postnatal arm were similar to the cord blood arm (Figure 2). In total, 39 infants had peripheral blood samples at 1 week, 40 infants at 1 month, and 28 infants at 36 weeks. The median age at blood collection for the three timepoints was similar between groups (Figure 2). Reasons for missed samples included incomplete recovery of scavenged blood, either because no sample was drawn at that timepoint for clinical indications or there was no remaining sample available for the study. Regardless, the rates of BPD did not differ significantly among infants with all three versus those with less than three consecutive samples. As listed in Supplemental Table S2, postnatal adductomics profiles were similar to cord blood in composition and range of concentrations. All 105 adducts that were identified in cord blood were detected in peripheral blood, with 49 unknown and 56 previously annotated adducts.

Figure 6 shows heatmaps of relative adduct concentrations at the three serial timepoints. Leading up to 1 week, non-significant trends of increasing adduct levels with increasing CSO appeared independent of BPD status. At 1 month, 51 known adducts were associated with high versus low CSO (fourth quartile versus first quartile; at least 2.1 FC; $P_{adj} < 0.05$) (Figure 6B). Cys^{34}→Oxoalanine (A004)—the adduct identified in cord blood with the highest fold-increase with BPD—was increased three-fold with high CSO exposure at 1 month ($P_{adj} < 0.05$). At 36 weeks, nine known adducts were significantly decreased with high CSO (Figure 6C). Of these, Cys^{34} sulfinic acid (A012)—the adduct decreased in cord blood with BPD-PH—was decreased with high CSO at 36 weeks ($P_{adj} < 0.05$).

In contrast to cord blood, there were no previously annotated adducts associated with BPD at any of the three timepoints. However, several unknown adducts correlated with CSO and BPD. Heatmaps of the relative concentrations of unknown adducts (Figure 7A) illustrate the high variability among individual patients across time with CSO exposure and BPD status. At 1 week, 48 unknown adducts were decreased with high CSO (fourth quartile; at least 2.5 FC; $P_{adj} < 0.05$). At 1 month, 24 unknown adducts were increased with high CSO (≥4.3 FC; $P_{adj} < 0.001$). Lastly, 45 unknown adducts directly correlated with BPD at 1 month (≥1.1 FC; $P_{adj} < 0.05$; Figure 7B) but none were correlated at 1-week or 36-weeks PMA.

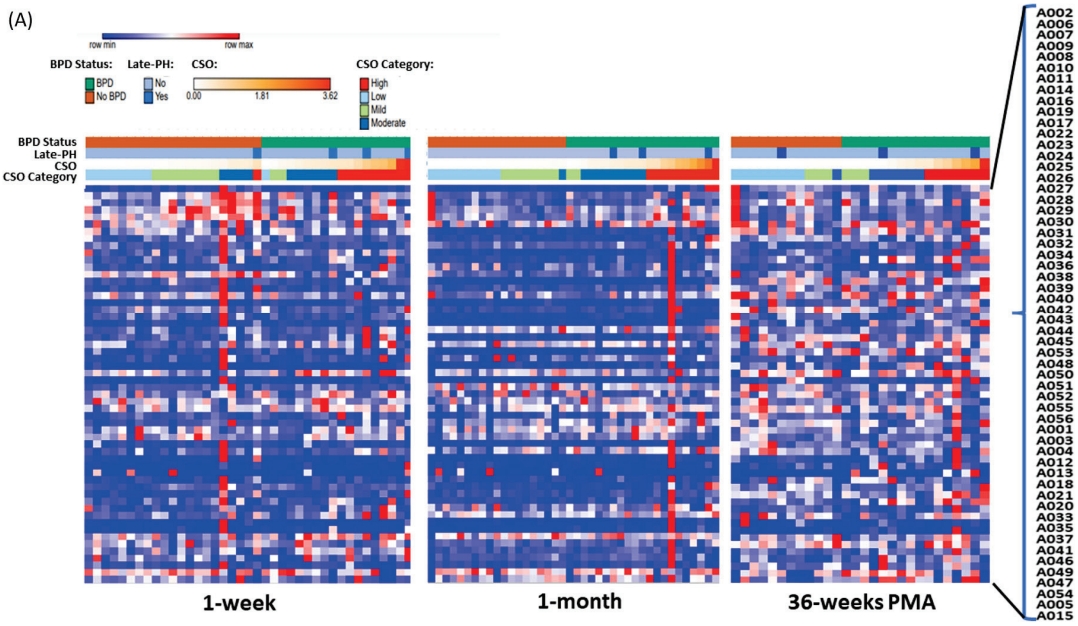


Figure 6. *Cont.*

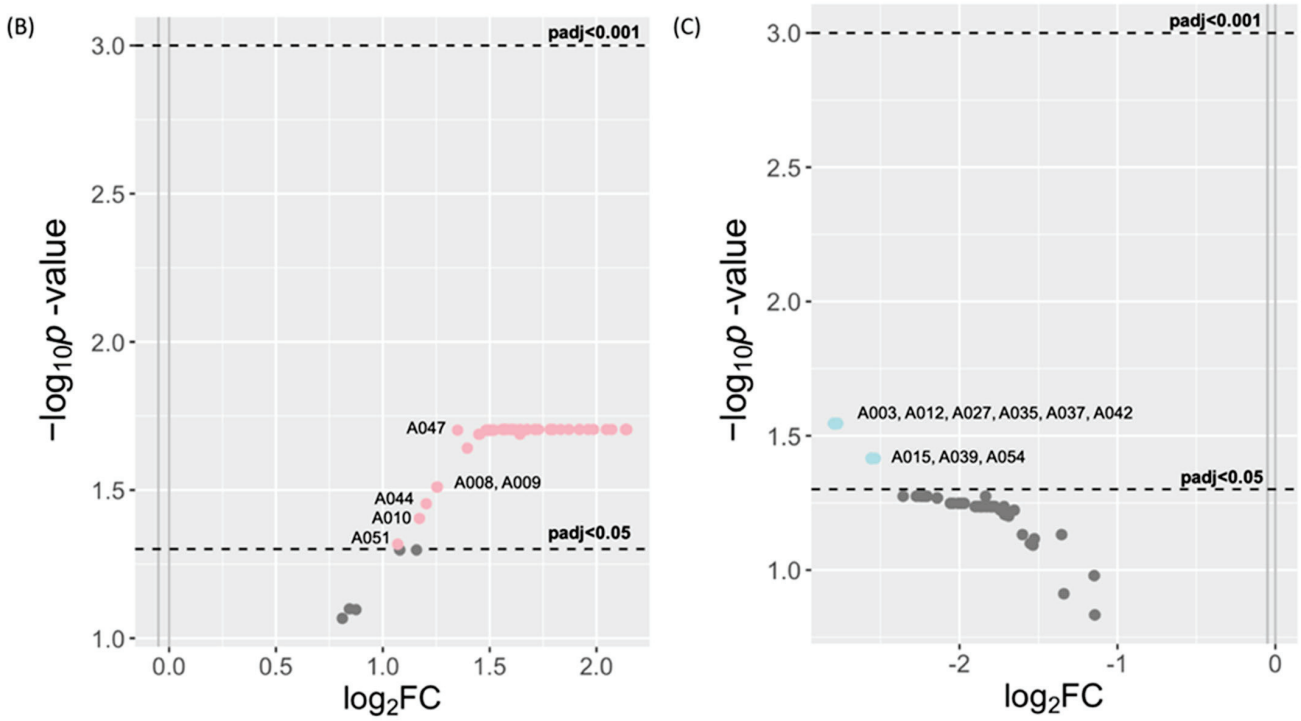


Figure 6. Known adducts detected in peripheral blood with cumulative supplemental oxygen (CSO) exposure and BPD. (**A**) A heatmap illustrating the variation in previously annotated adduct concentrations according to BPD and no BPD, and levels of CSO preceding 1-week, 1-month, and 36-weeks PMA. (**B**) At 1 month, there were 51 known adducts (red) associated with high CSO compared to low CSO (4th quartile versus 1st quartile, respectively; at least 2.1 FC; $P_{adj} < 0.05$). (**C**) At 36 weeks, 9 adducts were significantly decreased (blue) with high CSO exposure. Grey dots indicate adducts that were not significant ($p \geq 0.05$).

(**A**)

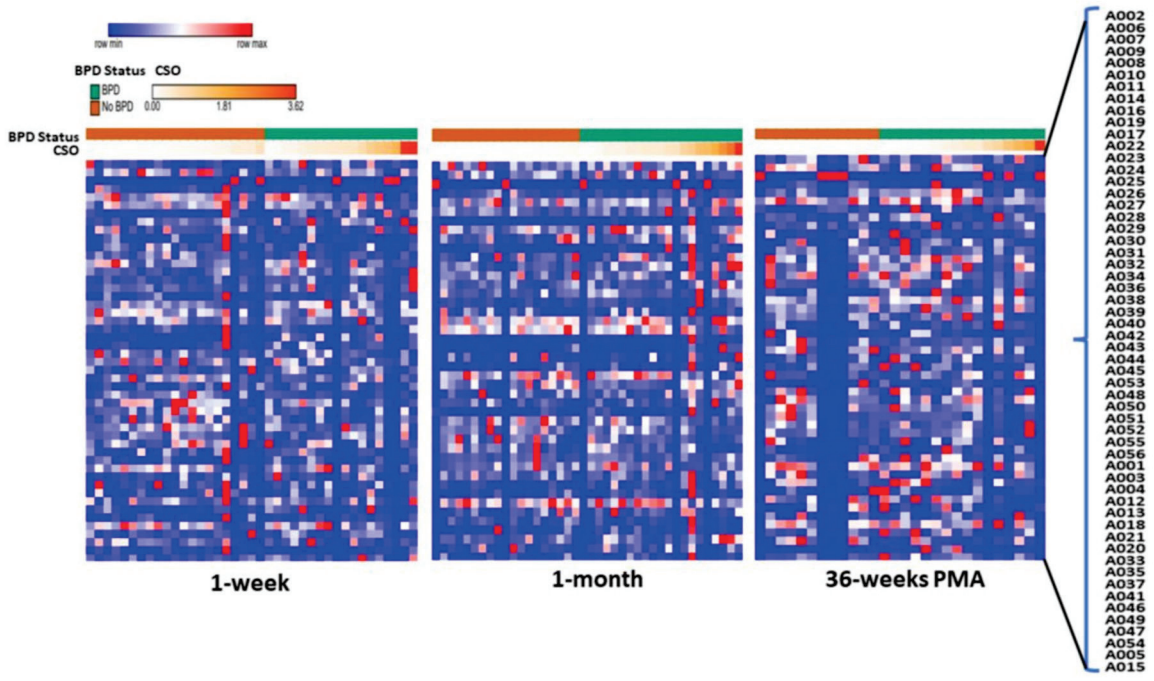


Figure 7. *Cont.*

(**B**)

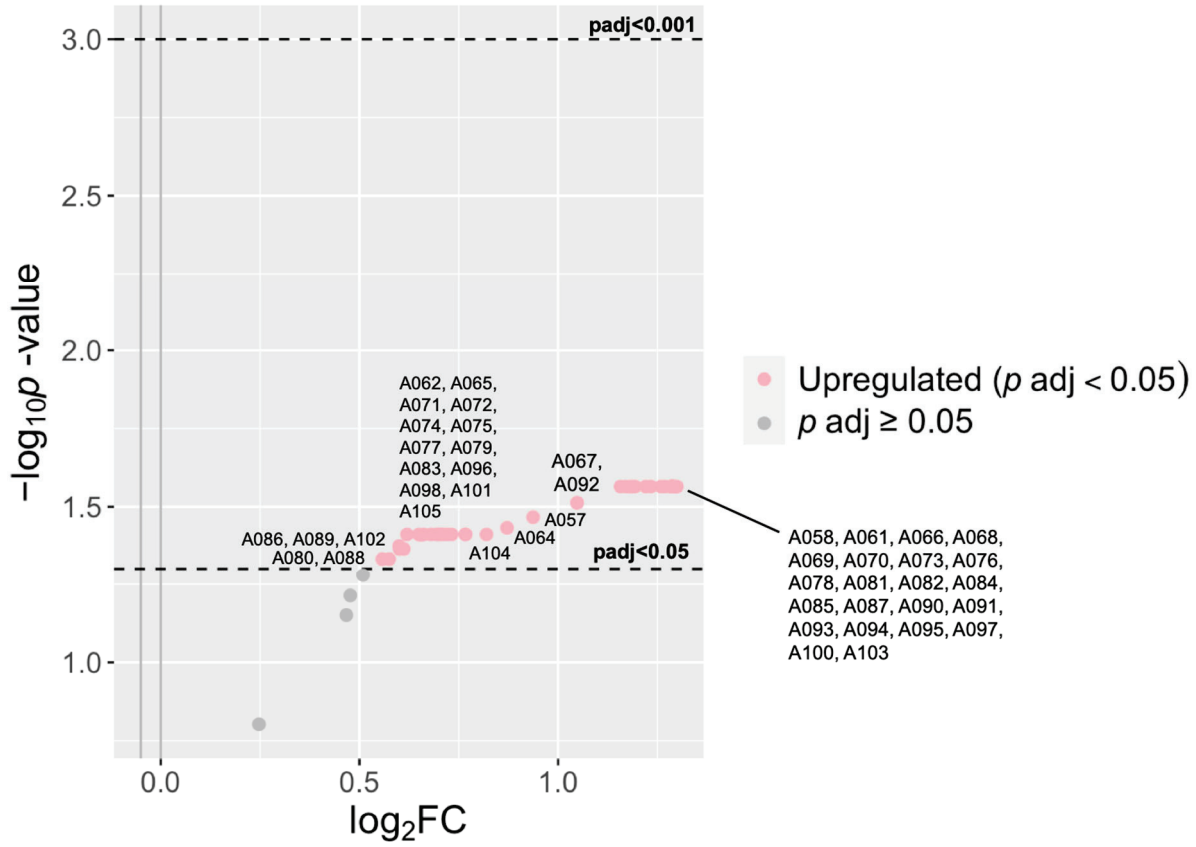


Figure 7. Unknown adducts in peripheral blood with CSO and BPD. (**A**) A heatmap showing the relative concentrations of unknown adducts according to BPD and CSO levels. In contrast to known adducts of oxidative stress, there was high variability among individual patients across time with CSO exposure and BPD status. (**B**) The volcano plot of the 49 unknown adducts at 1 month illustrates that 45 were significantly increased with BPD. Unknown 202.02 Da (A084) and Unknown 183.02 Da (A081) had a 2.5-fold increase with BPD ($P_{adj} < 0.05$).

4. Discussion

We employed novel adductomics approaches to identify protein adducts in cord blood and peripheral blood associated with extreme prematurity and BPD. The adducts included in this analysis are implicated in intrinsic and extrinsic pathways of OS, based upon their distinct biochemical structures. In total, 105 adducts were identified, with certain adducts varying in concentration that correlated with the degree of prematurity, the development of BPD, and exposure to supplemental oxygen. These included thiols, direct oxidation products, and others which have been associated with environmental exposures and disease states such as tobacco smoke, air pollution, and certain cancers [27,32,37,38]. While the literature on the role of adductomics in understanding the human exposome is rapidly expanding, this is the first study to investigate the "neonatal–perinatal exposome" of extremely preterm infants to identify exposure biomarkers of BPD.

Notable findings from this study include the following: (1) unmodified T3 was increased in cord blood with EPT; (2) cord blood $Cys^3 \rightarrow$Oxoalanine was increased with BPD; (3) Cys^{34} sulfinic acid was decreased with BPD-PH, but increased with pre-eclampsia; (4) in postnatal blood, several known adducts correlated with CSO, and several unknown adducts were increased with BPD. Heatmaps of relative concentrations suggest a trend toward lower levels of OS-related adducts in patients with pre-eclampsia and pulmonary hypertension, and increased levels with chorioamnionitis.

Elevated OS is an important mediator in the pathogenesis of BPD. Relative hyperoxia at birth and in the neonatal period, due to supraphysiologic oxygen exposure, generates ROS/RNS that damage the immature developing lung, inhibit growth and repair, and lead to chronic inflammation and fibrosis [39,40]. The reduced antioxidant capacity inherent in premature infants contributes to BPD pathogenesis [8]. Despite the proposed use of antioxidants in human infants [41], as well as widespread strategies to reduce supplemental oxygen exposure in the postnatal period [42,43], the incidence of BPD remains unacceptably high and variable among infants born at $\leq$28 completed weeks [41]. The association between BPD and early pregnancy risk factors such as IUGR now suggests that peripartum exposures, in particular those resulting in chronic fetal hypoxia of placental dysfunction and pre-eclampsia, play important roles and may be independent of postnatal factors such as hyperoxia [44,45]. These peripartum processes are poorly understood, largely because there are few stable biomarkers of OS at birth to elucidate these mechanisms.

Well-characterized OS biomarkers include byproducts of lipid peroxidation (8-isoprostane), oxidative DNA damage (8-hydroxy-2-deoxyguanosine), and others that capture the immediate biological state of ROS at the time of collection [46,47]. Unique to HSA is its nucleophilic hotspot to which a host of OS-related adducts binds. Unlike lipid peroxidation and DNA byproducts, HSA has a much longer half-life, with a mean residence time of about 1 month. Thus, HSA-Cys^{34} protein adductomics profiles from cord blood could serve as a molecular fingerprint of perinatal exposures in the weeks leading up to preterm delivery. These profiles captured at birth in cord blood, coupled with serial postnatal adductomics profiles in the weeks leading up to BPD diagnosis at 36-weeks PMA, provide the first report applying unbiased adductomics to a large cohort of NICU patients.

The patient sample represented a broad range of perinatal and postnatal characteristics, and included a sizable cohort of FT infants from a single center through which we could contrast the influence of extreme prematurity on adductomics profiles. Consistent with other preterm cohorts and the body of literature on BPD risk factors, lower GA and BW as well as SGA were associated with BPD (Table 1). Antenatal IUGR was a strong predictor of BPD, with 15.7% of BPD versus 1.9% of non-BPD infants having prenatally detected fetal growth restriction.

Two key adducts associated with BPD were Cys^{34}→Oxoalanine (A004) and Cys^{34} sulfinic acid (A012). A004, a protein truncation product that has been cited for its association with OS, was increased with BPD. In contrast, A012, a direct oxidation product, was significantly decreased with BPD-PH. In fact, the majority of known adducts increased with any BPD were decreased with BPD-PH. This suggests a dichotomy of OS pathways between BPD only and BPD-PH subphenotypes, in which BPD-PH may represent a more severe form of OS-mediated lung injury associated with a heightened antioxidant response in certain individuals, leading to a decrease in direct oxidation products bound to HSA-Cys^{34}. In contrast to BPD-PH, A012 was increased with pre-eclampsia—a specific peripartum risk factor for BPD-PH [25]. This "flip" from increase to decrease suggests that a transition in antioxidant capacity and the presence of direct oxidation products occurs, perhaps with heightened ROS/RNS generated by the placenta in the peripartum period [48] and subsequent upregulation of antioxidant activity in the infant during the postnatal period leading up to BPD at 36 weeks [49]. These adaptive mechanisms may be programmed during fetal development and necessary for survival, as suggested in the outlier case of an infant with BPD-PH with very high concentrations of OS-related adducts at birth who was exposed to intrauterine infection (chorioamnionitis) and eventually died at 36 weeks PMA (Figure 5A).

While the number of samples available for postnatal adductomics were relatively small, some trends were notable and consistent with the above speculations. At 1-month, but not at 1-week or 36-weeks PMA, a large proportion of known adducts were increased in infants with high CSO exposure in the weeks leading up to 1 month of age (Figure 6A). Again, there was a "flip" in the pattern in which only nine adducts varied according to CSO, and all nine were decreased in infants with high CSO in the weeks leading up to 36-weeks

PMA (Figure 6B). Of note, A012—the adduct which decreased with BPD-PH—was among those identified with high CSO.

Among the unknown adducts, a large majority were associated with high CSO and followed the trends of the known adducts in that they were largely decreased at 1 week, but increased at 1 month (Figure 7B). We speculate that a key transition in antioxidant activity may be occurring in the weeks preceding 1-month after birth that is mediated by CSO exposure, as 45 unknown adducts were increased at 1 month in BPD infants, but not at 1 week or 36 weeks.

There are currently over 50 previously annotated adducts described in adults [14,15,27,32–38] and over 40 unknown adducts recently identified [14,31–36,38,50]. The adducts identified in our study have been associated with pathological disease states: Cys^{34}→Oxoalanine is increased in individuals exposed to tobacco smoke, solid fuel, and benzene associated with colorectal cancer [37,38,51]. The unanticipated trend of decreased adduct levels in severe diseases such as BPD-PH has been observed in comparable human disease states. For example, in smokers, lower levels of direct oxidation products have been reported in association with smoking-induced hypoxia [14]. Cys34-sulfinic acid is decreased with air pollution exposure in chronic obstructive pulmonary disease (COPD) and ischemic heart disease (IHD) [33]. Liu and colleagues postulated that the increased OS of COPD and IHD generates greater production of Cys^{34} oxidation products. In turn, the hypoxia induced by severe lung disease, exacerbated by air pollution exposure, may dysregulate critical redox control pathways. Similar complex pathways may exist with perinatal chronic fetal hypoxia, postnatal hyperoxia, and severe BPD. For example, in cases of chronic fetal hypoxia associated with IUGR and placental insufficiency, the transition to relative hyperoxia at birth and in the postnatal period can trigger an OS cascade that overwhelms the compromised antioxidant system of the EPT infant and adapts to compensate for survival over time [8].

The majority of OS adducts were in small thiols, direct oxidation products, and reactive aldehydes groups (Figure 3). Small thiols, known for their antioxidant capacity, may be a response over time to high OS exposure. For instance, at 1 month of life, chronic high saturation of ROS from physiologic hyperoxia may activate antioxidant pathways. This is supported by the increase in *Cys^{34}→Oxalanine*, a protein truncation product resulting from OS, with high CSO at 1 month of life. Finally, reactive aldehydes are associated with lipid peroxidation, a process that triggers cellular dysfunction or intrinsic pathways, like apoptosis, in the setting of elevated OS. Ogihara and colleagues reported that reactive aldehydes are elevated at birth in premature infants who develop BPD. These aldehydes may induce the overexpression of cytokines involved in lung fibrosis [52].

The limitations of this study included the relatively small patient sample for the large number of adducts analyzed, and the single-center cohort design—which provided uniformity of the NICU environment, but future multi-center studies are needed to assess the generalizability of our findings. Nevertheless, this is the largest study of its kind, involving over 200 EPT infants. It was necessary to combine a pre-existing cohort with banked cord blood samples with a prospective cohort to follow serial timepoints and CSO exposures. There were some patients for which we could not recover data for all three timepoints. However, the same sets of adducts were identifiable and measurable in scavenged peripheral blood as in prospectively collected cord blood during the previous 13 years. This supports the stability of plasma adducts in archived samples and the future utility of HSA-Cys^{34} adducts as biomarkers for EPT infants as very minimal blood volume is needed (5 μL of sample for a single run). Recent studies of adductomics performed on neonatal dried blood spots [27,29] provide promise for the development of more practical approaches to tracking these exposure biomarkers over time in critically ill neonates. Utilizing dried blood spots has the advantage of being minimally invasive and more widely available as a source of peripheral blood measurement in neonates, with comparable stability in recent clinical studies [53–57]. Given that plasma and DBS samples are processed

very differently, further validation is needed in order to integrate adduct levels from different blood sources, timepoints, and collection methods.

Lastly, relative to other omics platforms in which biological pathway analyses are relatively more developed, the field of adductomics is still in its infancy. However, the findings of this study contribute to this rapidly growing field of exposure science. Validation studies employing synthetic chemistry work to characterize unknown adducts associated with BPD are important next steps. The investigation of known adducts in validation studies and experimental models is also warranted. Specifically, our understanding of the mechanistic links between developmental lung disorders such as BPD and specific maternal environmental exposures (e.g., toxins and pollutants), as well as other NICU exposures (e.g., diet and medications), could be enhanced by integrating adductomics into larger epidemiologic studies and future clinical trials. Additionally, HSA-Cys^{34} biomarkers can be utilized in diagnostics laboratories. For instances, Cys^{34} sulfinic acid (A012) can indicate the status of reactive oxygen species [27]. By training medical staff on how to pretreat blood samples (e.g., plasma or DBS), certain adducts, including Cys^{34} sulfinic acid (A012), can be used to measure oxidative-stress status.

5. Conclusions

In conclusion, the application of adductomics to elucidate exposure pathways of preterm birth is a novel and promising next step in BPD research. These exposure pathways could inform the development of antioxidant strategies to reduce the incidence and severity of BPD. Linked to other high-resolution and high-throughput technologies, investigating multiple omics could yield unprecedented findings and lead to innovative approaches in the identification, classification, and management of BPD and its subphenotypes. This study highlights the central and complex role of OS signaling and regulation in the pathogenesis of multi-factorial BPD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox13040494/s1, Table S1: Putative HSA-Cys^{34} adducts in cord and peripheral blood plasma; Table S2: Median of normalized HSA-Cys^{34} adduct concentrations according to health condition and collection timepoint; Figure S1: Calibration curves used to estimate adduct concentrations in pmol/mg.

Author Contributions: E.T.L. and Y.B. contributed to the study design, assays and data analysis, interpretation of results, and drafting of the initial manuscript. R.B., A.M.S. and R.Z. contributed to the study design, and sample and data acquisition. A.M.S. and K.M.F. designed and conducted the data analysis. W.F. and K.K.M. conceived and initiated the original study design, provided oversight of patient recruitment, assays, data collection, and analyses, and drafted this manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards of Northwestern University (protocol number: STU00201858) and Lurie Children's Hospital of Chicago (protocol number: 2019-3040).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the cord blood arm of the study. Patient consent was waived for the postnatal arm of the study under a scavenged biospecimen protocol instituted by the IRB during the COVID-19 pandemic.

Data Availability Statement: The raw adductomics data and the coded (deidentified) clinical data generated in this study are available from the corresponding authors upon request.

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

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antioxidants

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Review

Oxidative Stress Induced by Antivirals: Implications for Adverse Outcomes During Pregnancy and in Newborns

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Abstract: Oxidative stress plays a critical role in various physiological and pathological processes, particularly during pregnancy, where it can significantly affect maternal and fetal health. In the context of viral infections, such as those caused by Human Immunodeficiency Virus (HIV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), oxidative stress may exacerbate complications by disrupting cellular function and immune responses. Antiviral drugs, while essential in managing these infections, can also contribute to oxidative stress, potentially impacting both the mother and the developing fetus. Understanding the mechanisms by which antivirals can contribute to oxidative stress and examination of pharmacokinetic changes during pregnancy that influence drug metabolism is essential. Some research indicates that antiretroviral drugs can induce oxidative stress and mitochondrial dysfunction during pregnancy, while other studies suggest that their use is generally safe. Therefore, concerns about long-term health effects persist. This review delves into the complex interplay between oxidative stress, antioxidant defenses, and antiviral therapies, focusing on strategies to mitigate potential oxidative damage. By addressing gaps in our understanding, we highlight the importance of balancing antiviral efficacy with the risks of oxidative stress. Moreover, we advocate for further research to develop safer, more effective therapeutic approaches during pregnancy. Understanding these dynamics is essential for optimizing health outcomes for both mother and fetus in the context of viral infections during pregnancy.

Keywords: oxidative stress; antivirals; pregnancy; adverse outcomes; pharmacokinetics; in vitro studies; therapeutic strategies; drug safety; maternal health; fetal development

1. Introduction

Antiviral medications are often prescribed during pregnancy to manage viral infections that could pose significant risks to the mother and fetus. As remarked by Money et al., these therapies are more recent compared to traditional antibiotics, particularly in the context of pregnancy, where long-term safety data are sparse [1]. This lack of data is partly due to the fact that during pregnancy, individuals are often excluded from clinical trials, leaving a gap in understanding regarding the full effects of these medications during pregnancy [2]. As a result, several novel drugs may not be recommended for use in pregnant individuals since their safety has not been adequately studied in this population [3]. When considering treatment options, it remains important to prioritize up-to-date information to ensure safe care for the mother and the fetus. Avoiding unnecessary medications during the first trimester is still advisable, and careful consideration and judicious use of these therapies in later pregnancy is essential. The potential risks of inadvertent drug exposure

due to unplanned pregnancies must be weighed against the need for effective treatment, particularly given the uncertainties surrounding newer medications [4]. Drug exposure affects the fetus differently depending on gestational age, with examples including nonsteroidal anti-inflammatory drugs (NSAIDs) causing ductus arteriosus constriction in early pregnancy, teratogenic agents leading to malformations during organogenesis, beta-2 agonists [5] and selective serotonin reuptake inhibitors (SSRIs) influencing fetal heart function in later stages [6], and corticosteroids and warfarin [7] having varying impacts depending on the timing of exposure [8,9]. Determining safe treatments requires balancing medical necessity with the risk of inadvertent early exposure due to unplanned pregnancies. This complexity is intensified by the potential for antivirals to induce oxidative stress, which can have harmful effects.

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and the body's capacity to neutralize them, leading to cellular and tissue damage. During pregnancy, this can impact both maternal and fetal health, potentially resulting in complications such as preeclampsia, intrauterine growth restriction, and preterm labor [10,11]. Pregnancy itself is associated with increased oxidative stress due to heightened metabolic demands and physiological changes. The placenta, which plays an essential role in the signaling and regulation of cellular processes, is a major source of ROS [12]. Viral infections can exacerbate oxidative stress during pregnancy (Figure 1). For example, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection triggers a systemic inflammatory response, which can release pro-inflammatory cytokines [13], leading to uncontrolled infection, lymphocyte depletion, and increased tissue damage [11]. This inflammatory state is associated with increased oxidative stress, as inflammation can enhance the production of ROS. Preliminary findings by Mandò et al. suggest that the placentas of mothers infected with SARS-CoV-2 exhibit reduced levels of mitochondrial DNA (mtDNA) and altered expression of genes involved in mitochondrial function, particularly those related to mitochondrial dynamics and respiratory chain activity. Mitochondrial dysfunction in these placentas can impair cellular energy production and increase ROS generation, further exacerbating oxidative stress [14]. The body's defense against oxidative stress relies on a complex network of enzymatic antioxidants. Key enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) play critical roles in neutralizing ROS. For instance, SOD catalyzes the dismutation of superoxide radicals, while CAT breaks down hydrogen peroxide into water and oxygen [15]. During pregnancy, these enzymatic mechanisms become even more crucial. Peroxiredoxins and thioredoxin reductase contribute additional layers of cellular protection, helping to mitigate the increased oxidative stress associated with physiological changes and potential viral infections like SARS-CoV-2 [16]. The reduced expression of antioxidant defense genes, such as CAT and GSS, as observed in SARS-CoV-2-infected placentas further underscores the importance of these enzymatic systems in maintaining cellular integrity during pregnancy. This reduction compromises the placenta's ability to neutralize ROS, intensifying oxidative stress. The acute effects of viral infection, coupled with potential oxygen desaturation and placental malperfusion, may hinder the placenta's ability to activate compensatory mechanisms normally employed to mitigate oxidative damage [14]. There is evidence that the placenta plays a protective role in preventing fetal infection with SARS-CoV-2, and recent research has indicated that SARS-CoV-2 infection during the third trimester does not necessarily lead to significant changes in placental histology compared to controls, suggesting that the timing and duration of exposure may influence the placenta's response [17,18]. While there is growing evidence linking SARS-CoV-2 infection to oxidative stress and adverse pregnancy outcomes, including impacts on both the mother and neonate, this relationship is still being actively researched. The exact mechanisms by which SARS-CoV-2-induced oxidative stress contributes to these outcomes remain unclear, and more studies are needed to fully understand the interplay among viral infection, oxidative stress, and placental function during pregnancy.

Given the widespread use of antivirals during pregnancy, it is crucial to explore how these drugs might induce oxidative stress and the implications for maternal and neonatal health. Antiviral drugs are crucial for managing viral infections, yet their effects on oxidative balance in pregnancy are not fully understood. Some antiviral medications may contribute to oxidative stress, potentially exacerbating the already elevated levels associated with pregnancy and viral infections [19]. This review aims to (i) provide a comprehensive analysis of the mechanisms by which antivirals may trigger oxidative stress, (ii) evaluate the potential risks associated with their use during pregnancy, and (iii) highlight the need for further research to ensure the safety of mothers and their newborns.

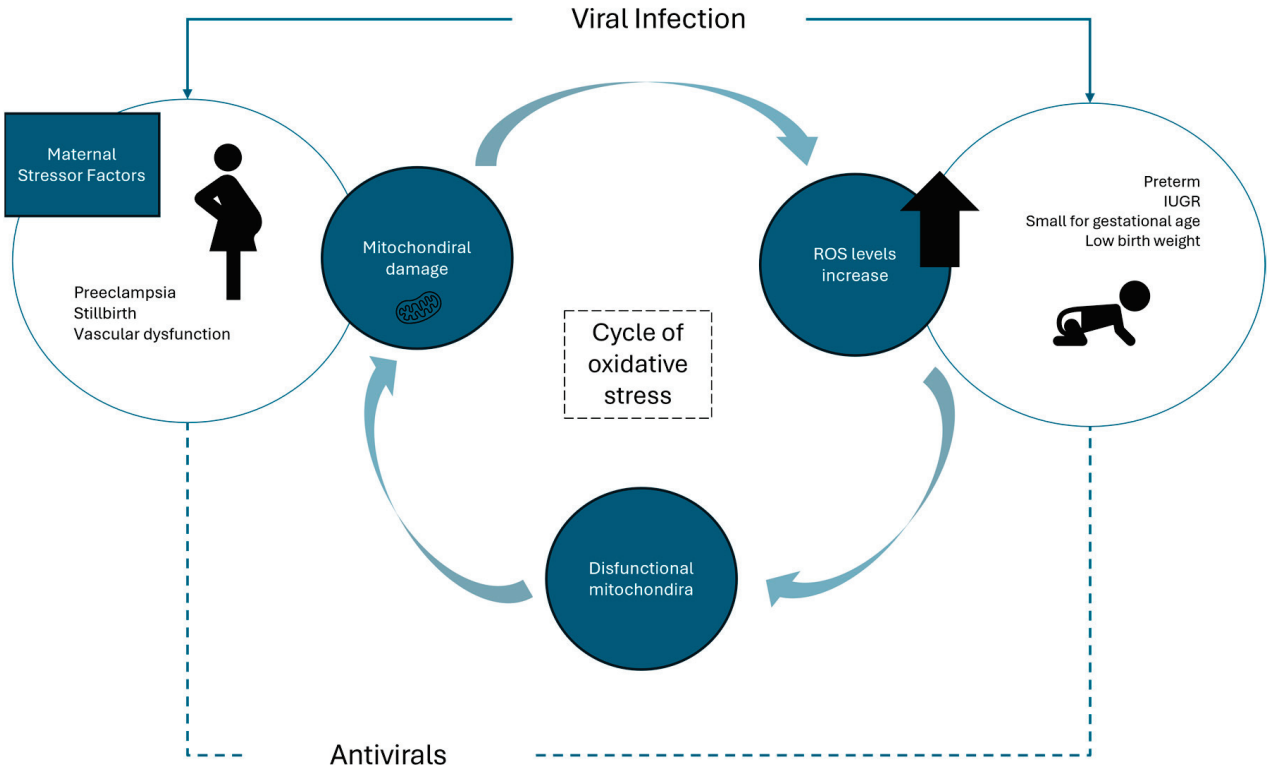


Figure 1. Oxidative stress is a mechanism for adverse outcomes for pregnant and newborns. Viral infections and the use of antivirals/antiretrovirals are associated with oxidative stress, which can have significant implications for maternal and neonatal health (e.g., intrauterine growth restriction (IUGR)). Image adapted from Nüsken et al. [20].

2. Viral Infections and Their Role in Oxidative Stress

Virus-induced toxicity involves complex mechanisms that lead to cellular damage and tissue dysfunction, with oxidative stress playing a central role. Understanding these mechanisms is crucial for developing targeted antiviral therapies and improving treatment outcomes. During viral replication, ROS are produced, causing oxidative damage to lipids, proteins, and DNA, which can result in cellular dysfunction or death. Although host cells activate antioxidative defense systems to restore redox balance, excessive ROS generated by viral activity and immune responses can result in tissue damage and inflammation. Viruses can alter host metabolism and modulate cellular processes, contributing to toxicity by manipulating mitochondrial functions to increase ROS levels [21,22]. Paradoxically, this can support viral replication while simultaneously triggering host antiviral responses. This interaction may also disrupt mitochondrial dynamics, such as morphology and membrane potential, which are critical for ROS production [23]. For instance, SARS-CoV-2 has been shown to manipulate mitochondrial functions to increase ROS levels. This manipulation helps the virus replicate more efficiently while triggering the host's antiviral responses [19].

This increased ROS production contributes to the severe inflammatory responses seen in COVID-19 patients, often leading to acute respiratory distress syndrome (ARDS) and other complications [24]. Hepatitis C virus (HCV) is another example where viral manipulation of mitochondrial functions plays a crucial role [25]. HCV infection leads to increased ROS production, which can cause liver inflammation and fibrosis [26]. The virus alters mitochondrial dynamics and disrupts the electron transport chain, leading to increased production of ROS. These ROS, along with pro-inflammatory cytokines, activate hepatic stellate cells, which play a crucial role in liver fibrosis [27]. Once activated, hematopoietic stem cells (HSCs) transform into myofibroblast-like cells that produce extracellular matrix (ECM) components, leading to fibrosis. After that, an inflammatory response is triggered, releasing cytokines such as tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), and transforming growth factor beta (TGF-β). These cytokines further enhance ROS production and perpetuate the cycle of inflammation and fibrosis [28,29]. Interestingly, the oxidative environment created by ROS also supports HCV replication. The virus benefits from oxidative stress to maintain its lifecycle, thereby sustaining chronic infection and ongoing liver damage [30].

The influenza virus induces inflammatory responses through several mechanistic pathways, primarily by increasing intracellular ROS levels, which disrupt the redox balance in host cells. During infection, ROS are generated as byproducts of mitochondrial metabolism, and elevated ROS production leads to oxidative stress, causing cellular damage. This imbalance promotes viral replication by compromising the host's immune defenses and enhancing viral entry and replication within cells. Additionally, the accumulation of ROS triggers programmed cell death (apoptosis) and stimulates the release of proinflammatory cytokines and chemokines, such as interferons (IFNs), TNFs, and interleukins (ILs) [31]. This cytokine storm exacerbates tissue damage, particularly in the lungs, leading to severe respiratory symptoms and contributing to the pathogenesis of influenza. The virus also hijacks host cell signaling pathways, such as the JNK/ERK/p38 MAPK and NF-κB pathways, which are activated by ROS, amplifying inflammation and lung injury [31,32]. Thus, the interplay among viral replication, oxidative stress, and the immune response creates a cycle in which ROS-induced tissue damage both facilitates viral replication and triggers a harmful inflammatory response, hindering the host's recovery without intervention. Not surprisingly, human immunodeficiency virus (HIV) infection is also associated with increased oxidative stress due to the virus's ability to manipulate host cell signaling pathways. The virus activates NADPH oxidase, leading to elevated ROS levels [33], contributing to the chronic inflammation and immune activation seen in HIV-infected individuals. Additionally, increased ROS levels can damage several tissues conducive to the development of comorbidities such as cardiovascular disease [34].

There are several cellular defense mechanisms against oxidative stress, including the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. Under normal conditions, Nrf2 is kept in the cytoplasm and degraded [35]. However, in response to oxidative stress, Nrf2 is released and translocated to the nucleus, activating the transcription of various antioxidant genes. These genes encode proteins that help detoxify ROS and protect cells from oxidative damage. Thus, the Nrf2 pathway plays a vital role in enhancing the host's antioxidative response to counteract virus-induced oxidative stress [36]. Interestingly, viruses can exploit this pathway to their benefit. Some viruses can enhance the Nrf2 pathway to promote their replication (positive modulation). For instance, certain viral proteins can induce oxidative stress that activates Nrf2, leading to the increased expression of antioxidant genes that may support viral metabolism. Conversely, other viruses may suppress the Nrf2 pathway to evade the host's antioxidative defenses (negative modulation) [37,38]. HBV and HCV can disrupt Nrf2 signaling pathways, contributing to liver damage and promoting viral replication [39]. Influenza A virus can also interfere with Nrf2 signaling [40]. SARS-CoV-2 has been shown to dysregulate Nrf2 activity, which may contribute to the severe inflammatory responses seen in some patients [41]. The modulation of the Nrf2 pathway by viruses has significant implications for the progression of viral diseases [42]. When viruses positively regulate the Nrf2 pathway, it may help them thrive by creating a favorable

environment for replication. On the other hand, if a virus suppresses the Nrf2 pathway, it can lead to uncontrolled oxidative stress, resulting in cell death and exacerbating the disease [43].

During pregnancy, three major factors render viral infections particularly concerning. First, altered immune response: pregnancy induces changes in the immune system that may affect the response to viral infections. A significantly attenuated interferon (IFN) response exists in isolated peripheral mononuclear cells [44]. The adaptive immune responses may be altered, with some studies showing diminished induction of certain antibody responses during the later stages of pregnancy. Second, increased susceptibility and physiological adaptations during pregnancy, including immunological and endocrinological alterations, render pregnant individuals susceptible to certain viral and bacterial infections. Third, viral infections during pregnancy pose risks to the mother and the developing fetus. This includes risks of maternal morbidity, pregnancy loss, stillbirth, intrauterine growth restriction, preterm birth, neonatal death, and congenital abnormalities [45]. At last, it is necessary to consider placental involvement; the placenta can be affected by viral infections [46]. Viruses can replicate in placental cells, potentially disrupting placental function and increasing the risk of vertical transmission [47]. The interplay between viral infections, the altered immune response during pregnancy, and oxidative stress creates a complex environment that can significantly impact maternal and fetal health. Understanding these interactions is crucial for developing effective strategies to prevent and safely manage viral infections during pregnancy [48]. Future research should focus on elucidating the specific mechanisms by which different viruses interact with the maternal–fetal unit and how oxidative stress modulates these interactions.

During pregnancy, the Nrf2 pathway also plays a dual role in viral infections, offering protective and potentially harmful effects. The placenta, which is particularly vulnerable to oxidative stress during viral infections, may benefit from Nrf2 activation in placental cells, helping to protect against virus-induced damage and maintain placental function [49,50]. Nrf2 plays a crucial role in fetal development by protecting against oxidative stress; modulating its activity could potentially reduce the impact of viral infections on the fetus [51]. Therapeutic strategies targeting the Nrf2 pathway are currently being explored. Selective Nrf2 activators are being developed to enhance antioxidant defenses against virus-induced oxidative stress without promoting viral replication, but these compounds must be carefully designed to avoid negative effects on fetal development [52]. Similarly, delivery systems targeted to the infected tissues, or the placenta could maximize benefits while minimizing systemic effects [53]. The timing of intervention, influenced by the stage of pregnancy and timing of infection, is critical for ensuring the safety and effectiveness of Nrf2-targeted therapies. Considering individual genetic variability and specific viral strains, personalized approaches may be necessary. Understanding these dynamics is crucial for developing therapeutic strategies that target pathways to enhance the host's antioxidative response and mitigate viral pathogenesis.

3. The Link Between Oxidative Stress and Antiviral Agents

3.1. Impact of Antiviral Medications on Antioxidant Mechanisms

Oxidative stress arises from several mechanisms, such as mitochondrial dysfunction [54], glutathione depletion, lipid peroxidation, and inflammation. Many antivirals, particularly transcriptase inhibitors (NRTIs), disrupt mitochondrial function, increasing ROS production. Despite being effective in controlling viral replication, some antivirals can paradoxically amplify inflammatory responses, indirectly contributing to oxidative stress [55]. The effects of antivirals on oxidative stress are particularly concerning during pregnancy, as they can contribute to adverse outcomes like fetal growth restriction and preterm birth. Several approaches are being explored to address these major concerns: (1) Antioxidant supplementation and co-administration of antioxidants with antiviral therapy to counteract oxidative stress [56]. (2) Developing less toxic antivirals focused on maintaining efficacy while reducing their impact on cellular antioxidant systems [57]. How-

ever, pregnant individuals often lack access to these novel drugs due to limited safety data; there has not been enough time to fully assess their long-term effects during pregnancy [58]. (3) Personalized medicine: tailoring antiviral regimens based on individual oxidative stress profiles and genetic factors can also produce better outcomes [59,60]. More research is needed to ensure their safe use in this population.

Common antiviral medications include NRTIs, protease inhibitors, and neuraminidase inhibitors. NRTIs, like zidovudine and lamivudine, inhibit viral replication by incorporating themselves into viral DNA, causing chain termination [22]. Protease inhibitors, such as ritonavir, block the protease enzyme, which is essential for viral maturation [23], while neuraminidase inhibitors, like oseltamivir, prevent the release of new viral particles from infected cells [24]. While these drugs are effective in controlling viral infections, their mechanisms can inadvertently contribute to oxidative stress. Research has shown that antiretroviral therapies (ARVs), particularly protease inhibitors like ritonavir and saquinavir, can induce oxidative stress and neuronal damage, especially in the central nervous system. Studies demonstrated that ARVs can cause synaptic injury and neuronal death, with evidence of oxidative stress markers persisting in the brains of patients on combination antiretroviral therapy (cART), even when viral replication is controlled. This suggests that ARVs control infections but may also contribute to neuronal damage through oxidative stress mechanisms [25].

Zidovudine and lamivudine have been associated with mitochondrial toxicity, leading to increased ROS production due to impaired electron transport chain function [26]. This can result in oxidative damage to mitochondrial DNA, proteins, and lipids, which may exacerbate cellular dysfunction [27]. The drug efavirenz, which inhibits reverse transcriptase by binding to a site distinct from NRTIs [28], has been associated with liver damage (hepatotoxicity) that may be partly caused by oxidative stress mechanisms [29]. Protease inhibitors can disrupt lipid metabolism, increasing free fatty acid levels and oxidative stress and potentially inducing endoplasmic reticulum (ER) stress, triggering the unfolded protein response and increasing ROS production [30,31]. These oxidative effects are particularly concerning during pregnancy, as they could possibly affect fetal development and maternal health [32]. Neuraminidase inhibitors like oseltamivir, primarily used against influenza viruses, prevent the release of newly formed viral particles from infected cells [33]. Generally, they have fewer oxidative stress-related side effects compared to NRTIs, and protease inhibitors have suggested potential oxidative effects. Evidence indicates that neuraminidase inhibitors may influence cellular redox signaling pathways, potentially affecting oxidative stress levels [34,35]. Integrase inhibitors, generally well-tolerated, have been reported to cause mitochondrial dysfunction. Interestingly, raltegravir and dolutegravir increase oxidative stress and mitochondrial dysfunction while simultaneously promoting lipogenesis and adipogenesis [36]. Moreover, it has been demonstrated that dolutegravir increases cellular mass, mitochondrial ROS, and apoptosis and disrupts mitochondrial networks in human fibroblast WI-38. The effects of integrase strand transfer inhibitors are still not fully understood, and additional research in cohorts with female participants to support the action of hormones on mitochondria is necessary to determine whether there are any alterations in mtDNA or biogenesis [37].

Antivirals used for COVID-19 and other viral infections have demonstrated variable effects on oxidative stress [61]. Remdesivir is demonstrated to be effective against SARS-CoV-2; however, studies indicate that it may increase oxidative stress in liver cells, raising concerns about potential hepatotoxicity [62,63]. Similarly, ganciclovir, used in the treatment of cytomegalovirus (CMV) infections, has been shown to induce oxidative stress in certain cell types, potentially contributing to its toxicity profile [61]. Its prodrug, valganciclovir, may exhibit comparable oxidative effects due to its conversion to ganciclovir in vivo. In contrast, tenofovir, widely used for hepatitis B management, is generally well-tolerated, with limited direct evidence suggesting a significant impact on oxidative stress. This positions tenofovir as a relatively safer antiviral option in terms of oxidative balance [64,65]. Direct-acting antivirals for Hepatitis C, while highly effective in achieving viral clearance,

have been reported to temporarily increase oxidative stress markers during treatment in some studies [62]. This transient oxidative stress may not have long-term adverse effects but warrants consideration in specific patient populations [66]. Finally, protease inhibitors are associated with increased oxidative stress, which contributes to metabolic complications such as insulin resistance and dyslipidemia [63]. These findings underscore the need for the careful monitoring of oxidative stress and metabolic health in patients receiving protease inhibitor-based therapies [63]. These findings underscore the need for careful monitoring of oxidative stress and metabolic health in patients receiving protease inhibitor-based therapies [67].

Antiviral drugs can contribute to oxidative stress and, by contrast, might have minimal impact on oxidative stress or even possess antioxidant properties. For example, umifenovir (arbidol), an antiviral drug used against influenza, has demonstrated antioxidant potential through the inhibition of lipid peroxidation and the prevention oxidative damage to the mitochondrial respiratory chain [68]. The drug exhibits antiviral and antioxidant effects, potentially protecting cells from oxidative stress induced by viral infections. These features might influence cellular redox signaling pathways, diminishing their impact on oxidative stress in comparison with other NRTIs or protease inhibitors. Similarly, silymarin, echinichrom A, and arctigenin also possess both activities. Silymarin has shown potent antiviral activity against the Mayaro virus and antioxidant activity by reducing biomarkers of oxidative stress [69]. Echinochrome A, when combined with ascorbic acid and alpha-tocopherol, demonstrated higher antioxidant and antiviral effects than echinochrome A alone [70]. Arctigenin has shown both antioxidant and antiviral activities against Japanese encephalitis virus (JEV) infection [71].

To address oxidative stress concerns with antiviral therapy, it is possible to (a) combine drugs with antioxidants to counteract oxidative effects. For example, interferon alpha-2b combined with unithiol showed synergistic antiviral effects against Herpes simplex virus [72]; (b) develop dual-action drugs creating antiviral compounds with inherent antioxidant properties; (c) administer antioxidants alongside antiviral therapy to mitigate oxidative stress or even consider non-thermal plasma (NTP) therapy. This emerging approach manipulates oxidative stress responses to treat viral infections like herpes simplex virus type 1, potentially offering an alternative to traditional antiviral drugs [73].

Antiviral medications are essential for managing viral infections; nevertheless, a deeper understanding of their interference with antioxidant mechanisms is required. This will provide better monitoring and the development of supportive strategies to minimize adverse effects, especially in vulnerable populations like pregnant. Most studies have focused on in vitro or animal models, with limited data available from human studies. There is a lack of comprehensive studies evaluating the long-term effects of antiviral-induced oxidative stress on both maternal and neonatal outcomes. Additionally, the potential for antioxidant therapies to mitigate these effects has not been adequately explored. Addressing these gaps is essential for developing safer antiviral treatment protocols during pregnancy and reducing the risk of adverse outcomes for both mothers and their newborns.

3.2. Challenges in Assessing Oxidative Stress Caused by Antivirals

Assessing oxidative stress caused by antivirals presents several challenges, including the complexity of accurately measuring ROS and their impact on biological systems. For example, ROS levels fluctuate rapidly, making it difficult to capture real-time oxidative stress [74]. Furthermore, the effects of antivirals on oxidative stress vary based on the drug class, dosage, duration of treatment, and individual patient factors such as genetics and underlying health conditions. Another challenge is distinguishing oxidative stress directly induced by the antiviral itself from viral infection or inflammation effects. The lack of standardized biomarkers to reliably assess oxidative stress in clinical settings complicates the evaluation of its long-term impact, especially in sensitive populations like pregnancy, where oxidative stress can contribute to adverse outcomes. Finally, the interplay between antiviral efficacy and oxidative damage increases complexity, as drugs designed to control

viral replication may inadvertently exacerbate oxidative stress [75]. Table 1 summarizes the challenges of assessing oxidative stress with antivirals. Usually, the body maintains a balance between ROS production and antioxidant activity through endogenous systems like SOD, CAT, and GPx. However, when this balance is disrupted, oxidative stress occurs, which can lead to inflammation, apoptosis, and tissue damage [76]. Since antivirals can induce ROS production through different mechanisms, it is challenging to pinpoint the exact source [77].

Table 1. Challenges in assessing oxidative stress related to antiviral treatments.

Challenges in Assessing Oxidative Stress with Antivirals		**Details**
Complexity of Oxidative Stress Mechanisms	Multiple ROS Sources	Antivirals can induce ROS production through different mechanisms (e.g., mitochondrial dysfunction, ER stress), making it challenging to pinpoint the exact source.
	Antioxidant System Variability	Individual differences in antioxidant defense systems can affect the overall oxidative stress response to antivirals.
Temporal Dynamics	Acute vs. Chronic Effects	Short-term oxidative stress may differ significantly from long-term effects, requiring longitudinal studies to capture the full impact of antiviral therapy.
	Adaptive Responses	The body may adapt to oxidative stress over time, potentially masking the true extent of damage in long-term assessments.
Measurement Challenges (Biomarker limitations)	Specificity	Many oxidative stress biomarkers lack specificity to particular ROS or antioxidants, making it difficult to attribute changes to specific antiviral drugs.
	Stability	Some oxidative stress markers are unstable and can be affected by sample handling and storage conditions.
	Methodological Issues	Invasiveness: Direct measurement of ROS often requires invasive procedures, limiting their applicability in clinical settings.
	Indirect Measurements	Many assessments rely on indirect markers of oxidative damage (e.g., lipid peroxidation products), which may not always accurately reflect the current oxidative state.
Confounding Factors	Underlying Disease State	The viral infection itself can induce oxidative stress, making it challenging to distinguish drug-induced effects from disease-related oxidative stress.
	Lifestyle Factors	Diet, exercise, and other lifestyle factors can significantly influence oxidative stress levels, potentially confounding the assessment of antiviral-induced effects.
Variability in Drug Responses	Pharmacogenomics	Genetic variations can affect how individuals metabolize antivirals, leading to differences in drug-induced oxidative stress.
	Drug Interactions	Many patients receive multiple medications, which can interact and affect oxidative stress levels in unpredictable ways.
Tissue-Specific Effects	Localized vs. Systemic Effects	Antivirals may induce oxidative stress in specific tissues or organs, which may not be reflected in systemic measurements
	Accessibility	Some tissues affected by oxidative stress may not be easily accessible for direct measurement.
Technological Limitations	In vivo vs. In vitro Discrepancies	Results from cell culture studies may not accurately reflect the complex in vivo environment.
	Real-Time Monitoring	Current technologies often lack the ability to provide real-time, continuous monitoring of oxidative stress in clinical settings.

4. Impact of Antivirals During Pregnancy and Adverse Outcomes in Newborns

4.1. Oxidative Balance During Pregnancy and Viral Infection

Oxidative stress is a physiological phenomenon during pregnancy, primarily arising from increased placental metabolism and oxygen demand. While oxidative stress plays a critical role in cellular signaling and fetal development, its effects are not exclusively harmful. Under normal conditions, the body's antioxidant defenses maintain a balance, allowing oxidative stress to support essential physiological processes. However, when this balance is disrupted and oxidative stress becomes excessive, it can lead to complications such as preeclampsia, IUGR, and preterm birth. Understanding this dual role is essential for evaluating the impact of external factors, such as viral infections and antiviral therapies, on maternal and fetal health [78].

Viral infections, such as SARS-CoV-2, can disrupt this balance, amplifying oxidative stress and inflammation and impair placental function [79]. The placenta, a critical organ in regulating maternal–fetal exchange and immune responses, is highly vulnerable to oxidative stress. In pregnant women infected with SARS-CoV-2, mitochondrial and oxidative imbalances in the placenta have been linked to adverse outcomes, such as placental dysfunction and impaired fetal development [14]. Research has shown that placental Nrf2 levels decrease in pregnant COVID-19-positive patients, especially those with symptomatic infections, and are associated with increased oxidative damage [80]. This mitochondrial dysfunction disrupts the placenta's ability to manage ROS, leading to heightened oxidative stress. The expression of key antioxidant genes and mitochondrial respiratory chain components is also reduced in COVID-19-affected placentas, further compounding oxidative damage [14,79]. The placental oxidative stress during pregnancy with COVID-19 may contribute to systemic endothelial dysfunction, impaired placental angiogenesis, and compromised transport mechanisms. These alterations can result in pregnancy complications such as preeclampsia, intrauterine growth restriction, and fetal developmental issues [81]. Iron metabolism is also significantly altered in these cases, with increased iron uptake and storage in the placenta contributing to oxidative stress through the Fenton and Haber–Weiss reactions [82]. Moreno-Fernandez et al. highlights an adaptive increase in certain antioxidant vitamins, such as vitamin D, E, and coenzyme Q10, in the placentas of COVID-19 patients. This increase is hypothesized as a compensatory mechanism to mitigate the oxidative stress induced by the viral infection. However, despite these adaptive responses, the overall oxidative imbalance can still have long-term effects on maternal and fetal health [79]. The findings emphasize the importance of understanding how viral infections, like SARS-CoV-2, affect the oxidative balance during pregnancy, as this can lead to immediate and long-term adverse outcomes for the newborn. Addressing this oxidative stress through appropriate interventions may be crucial in reducing the risk of pregnancy complications and improving fetal development.

Several viruses and consequent infections can affect oxidative balance during pregnancy in distinct ways, which may impact fetal growth and development. For instance, Influenza A virus (IAV) infection during pregnancy leads to the enhanced production of ROS by activated immune cells like macrophages and neutrophils and the depletion of antioxidant defenses, including enzymes and non-enzymatic antioxidants [83], triggering an exacerbated inflammatory response contributing to oxidative stress. The oxidative imbalance might lead to several maternal complications, such as (a) acute cardiopulmonary distress syndrome, increasing the risk of severe illness and hospital intensive care unit (ICU) admission; (b) vascular dysfunction that potentially contributes to a "Vascular Storm" that resembles preeclampsia [84]; (c) increased risk of secondary bacterial and viral pneumonia with higher morbidity and mortality compared to non-pregnant patients [85].

IAV infection-induced oxidative stress is associated with adverse pregnancy outcomes, namely, a higher risk of stillbirth, particularly during pandemic influenza seasons. Additionally, there is a potential increased risk of preterm birth (especially when fever symptoms exist) and reduced birth weight in full-term newborns. The impact of IAV infection on oxidative stress and pregnancy outcomes is variable based on the timing of infection, with

the highest incidence occurring during the first trimester. The major severity cases of the infection are associated with greater oxidative imbalance and worse outcomes [83]. In the case of HIV, the oxidative imbalance can increase the risk of opportunistic infections due to compromised immune function, potential exacerbation of HIV-related symptoms and disease progression, and higher susceptibility to pregnancy-related complications like preeclampsia. Studies have shown specific redox imbalance markers in HIV-infected pregnant patients [86]. Higher levels of malondialdehyde (MDA) indicate increased lipid peroxidation. Elevated activity of antioxidant enzymes like SOD and CAT suggests a compensatory response to increased oxidative stress. Changes in protein carbonylation levels indicate protein oxidation. Understanding the relationship between HIV infection, pregnancy, and oxidative stress has important implications, highlighting the need for close monitoring of HIV-infected pregnant patients for signs of oxidative stress-related complications [87].

HBV and HCV infections are responsible for an increased risk of gestational diabetes, particularly with HBV infection [88,89]. Higher susceptibility to pregnancy-related complications like preeclampsia, specifically HBV infection, is associated with a 10% increased risk of preeclampsia compared to uninfected individuals [90]. There is also a potential exacerbation of liver disease, especially in women with pre-existing chronic hepatitis. HBV and HCV infections can increase the susceptibility of placental cells to apoptosis and alter placental barrier function, potentially increasing the risk of vertical transmission [91]. Disruption of normal placental development and function may contribute to adverse pregnancy outcomes [92]. Oxidative stress from HBV and HCV infections during pregnancy may have long-term health implications for the offspring, including the potential increased risk of chronic liver disease in children infected perinatally and possible developmental issues related to prenatal exposure to oxidative stress [93].

CMV infection can disrupt placental function by increasing the susceptibility of syncytiotrophoblast cells to apoptosis [94,95]. CMV infection induces chronic lymphoplasmacytic villitis, characterized by inflammation of the placental villi. This inflammation can impair the placenta's ability to support the fetus [96,97]. CMV infection leads to increased levels of pro-inflammatory cytokines such as MCP-1 (CCL2) and TNF-α in the placenta. These cytokines can disrupt normal placental function and contribute to adverse pregnancy outcomes [98]. This virus can directly infect placental cells, causing cytopathic effects such as cell enlargement (cytomegaly) and the formation of intranuclear and intracytoplasmic inclusions; the direct damage can impair placental structure and function [97]. CMV infection can affect the function of cytotrophoblasts, which are crucial for the formation of anchoring villi that attach the placenta to the uterine wall. This impairment can reduce the size and function of these villi, compromising placental attachment and nutrient exchange [99]. These histopathological changes and elevated levels of MCP-1 and TNF-α in the placenta are indicative of significant placental damage and dysfunction and are associated with adverse pregnancy outcomes such as fetal growth restriction and preterm birth. Ex vivo studies using placental explants have shown that CMV infection can significantly increase pro-inflammatory cytokine expression, further supporting cytokine dysregulation's role in CMV-induced placental dysfunction [99].

Understanding the relationship between viral infections and oxidative stress during pregnancy is crucial for developing targeted interventions. Ongoing research into antioxidant therapies, immune modulation, and tailored care aims to mitigate viral-induced oxidative stress. Balancing the immune response and reducing inflammation could help protect against oxidative damage. Since each virus disrupts oxidative balance differently, strategies such as strict control of oxygen administration, use of antioxidants like lutein and melatonin, and hypothermia to mitigate these risks show promise as potential neuroprotective interventions [100,101]. Further research is essential to prevent and manage these complications effectively.

4.2. Pre-Existing Maternal Health Conditions and Antiviral Therapy

Maternal pre-existing conditions, such as diabetes, hypertension, obesity, and chronic viral infections, can amplify the oxidative stress burden during pregnancy, creating a complex interplay between maternal health and antiviral therapy. These conditions are often associated with elevated baseline levels of ROS and reduced antioxidant defenses, increasing susceptibility to oxidative damage [102].

Antiviral drugs, while essential for managing maternal viral infections, may further exacerbate oxidative stress. Increased oxidative stress can worsen glycemic control, leading to further complications such as gestational diabetes or preeclampsia. The accumulation of free radicals from antiviral therapy may impair vascular function, increasing the risk of placental dysfunction and preeclampsia. Obesity-related oxidative stress, combined with drug-induced ROS production, could heighten systemic inflammation, impacting both maternal and fetal health [103].

The combined effect of pre-existing conditions and antiviral therapy-induced oxidative stress may accelerate disease progression. In mothers with chronic viral infections such as HIV or hepatitis, antivirals like NRTIs are known to cause mitochondrial dysfunction, which can worsen oxidative stress and heighten the risk of liver damage or cardiovascular disease. Chronic inflammation in these conditions, compounded by drug-induced ROS, could increase the risk of adverse outcomes such as preterm labor or fetal growth restriction [104].

Addressing oxidative stress in mothers with pre-existing conditions requires tailored therapeutic strategies. These may include the co-administration of antioxidants with antiviral therapy or the selection of antivirals with minimal oxidative effects. Personalized treatment plans based on the mother's health status and oxidative stress biomarkers can help mitigate risks, ensuring better maternal and fetal outcomes.

4.3. Adverse Outcomes in Newborns Caused by Viral and Antiviral-Induced Oxidative Stress

Oxidative stress, resulting from an imbalance between pro-oxidant and antioxidant factors, poses significant risks to newborns, especially preterm infants [105]. At birth, newborns experience a sudden increase in oxygen availability, leading to enhanced free radical generation [106]. Preterm infants are particularly vulnerable due to immature antioxidant systems and reduced ability to control free radical overproduction [107]. Oxidative stress is implicated in various neonatal conditions, including bronchopulmonary dysplasia, retinopathy of prematurity, and intraventricular hemorrhage [108]. Oxidative stress is linked to respiratory conditions like bronchopulmonary dysplasia (BPD) and respiratory distress syndrome (RDS) in preterm infants. It can also contribute to brain injuries, potentially leading to long-term neurodevelopmental issues. Oxidative stress can exacerbate inflammation, increasing newborns' susceptibility to infections [105,109]. Advanced analytical techniques using mass spectrometry can help detect specific biomarkers for the improved diagnosis and treatment of oxidative stress-related conditions in newborns. The most relevant biomarkers of oxidative stress during the neonatal period include byproducts of oxidative damage to proteins, lipids, and DNA, and they are measurable in fluids like blood, urine, and amniotic fluid (Table 2). Oxidative stress biomarkers are crucial for assessing neonatal health, particularly in preterm infants who are more susceptible to oxidative stress-related complications [110]. Key biomarkers include isoprostanes, advanced oxidation protein products (AOPP), and non-protein-bound iron (NPBI), which reflect lipid, protein, and DNA oxidation, respectively [110]. These markers have shown diagnostic and prognostic value in various neonatal diseases [106,107]. Traditionally, blood samples were used for biomarker analysis, but there is a growing trend toward non-invasive sampling methods to reduce pain and excessive blood extraction in neonatal intensive care units [106]. Cord blood, urine, and saliva have been identified as valid and ethically acceptable biological samples for oxidative stress biomarker analysis in the perinatal period [106,107]. High-performance liquid chromatography–tandem mass spectrometry has been developed to accurately determine these biomarkers in non-invasively obtained biofluids [110]. Key

biomarkers include the glutathione/glutathione disulfide (GSH/GSSG) ratio, which reflects the redox status and is a reliable indicator of oxidative stress and it is measured in umbilical cord blood, whole blood, or tissue Biomarkers like the o-Tyr/Phe, m-Tyr/Phe, $3NO_2$-Tyr/p-Tyr, and 3Cl-Tyr/p-Tyr ratios, which indicate protein modifications due to oxidative stress, such as tyrosine nitration and chlorination, detectable in urine, plasma, or milk; the 8-oxodG/2dG ratio (used to detect oxidative DNA damage), commonly assessed in urine or plasma. Finally, biomarkers like isoprostanes (IsoPs), isofurans (IsoFs), and neuroprostanes (NeuroPs) indicate lipid peroxidation. Altogether, these biomarkers are critical for assessing oxidative stress in neonates, particularly in conditions like hypoxic–ischemic encephalopathy [110]. Oxidative stress during pregnancy can impact fetal development, leading to various adverse outcomes, including neural tube defects, neurodevelopmental disorders, and long-term metabolic syndromes. Research demonstrates that oxidative stress contributes to conditions like respiratory distress syndrome, bronchopulmonary dysplasia, necrotizing enterocolitis, and retinopathy of prematurity [111]. Antivirals, although necessary to control viral infections during pregnancy, can inadvertently trigger these oxidative stress pathways, further complicating neonatal outcomes. In Table 3, we summarize the effect of antivirals on oxidative stress.

Influenza can significantly impact fetal development, primarily through oxidative stress, which may impair placental function and restrict nutrient supply, leading to IUGR. Despite the vertical transmission of IAV being rare, highly pathogenic strains like H5N1 may affect the placenta, and even low-pathogenic strains can cause placental apoptosis and replication issues, contributing to impaired function [112]. This placental dysfunction can result in a variety of adverse fetal outcomes, such as cleft lip, cleft palate, and congenital heart defects [113]. Seasonal influenza, for example, has been associated with increased risks of preterm birth, miscarriage, and fetal growth retardation (FGR), although research findings are sometimes inconsistent [114]. Studies indicate that IUGR and FGR are linked to reduced placental blood flow, limiting fetal oxygen and nutrient delivery [115]. Data from the 2009 influenza pandemic suggested increased risks of fetal death, small for gestational age (SGA) births, and respiratory illnesses in infants born to infected mothers [116,117]. In contrast, other studies have found no significant differences in fetal outcomes during influenza season, leading to debate over the direct impact of IAV on fetal growth.

IUGR resulting from placental dysfunction may also increase the risk of neurodevelopmental disorders such as schizophrenia. Placental inflammation and vascular impairment during maternal infections, compounded by conditions like pregnancy-induced hypertension (PIH) and preeclampsia, have been proposed to contribute to neurodevelopmental issues [118,119]. Animal models support this hypothesis, demonstrating behavioral and brain abnormalities similar to schizophrenia or autism in the offspring of IAV-infected mothers [120,121]. Recent systematic reviews have found no conclusive association between maternal IAV infection during pregnancy and schizophrenia, emphasizing the need for further research into the long-term neuropsychiatric impacts of maternal influenza infection [83]. For HIV, oxidative stress can similarly lead to IUGR, affecting placental function and increasing the risk of vertical transmission if left untreated. The disruption of placental development and function and damage to syncytiotrophoblast cells further contribute to complications. Long-term effects on fetal development and health are also a concern. Hepatitis B and C infections exacerbate the risk of preterm birth, with HBV being associated with a 17% higher risk of preterm delivery compared to uninfected individuals [90]. In addition, these infections can impair placental function, leading to IUGR and increasing the risk of vertical transmission, especially if proper immunoprophylaxis is not provided.

Similarly, CMV infection can result in severe developmental issues, including hearing loss, vision problems, and neurological deficits, with long-term consequences for the child's development and health [122]. CMV infection can disrupt normal placental function by increasing oxidative stress [123]. Elevated oxidative stress can damage placental cells, impairing nutrients and oxygen transfer to the fetus. Adverse neonatal outcomes cause

neurodevelopmental issues, including conditions like microcephaly, sensorineural hearing loss, and growth retardation. Oxidative stress can impair fetal growth, leading to IUGR [124]. Newborns with IUGR are smaller than expected for their gestational age and may face long-term health challenges [125]. CMV-induced oxidative stress can also affect the liver and spleen, leading to conditions like hepatosplenomegaly (enlarged liver and spleen), jaundice, elevated liver enzymes [126], and weaken the neonatal immune system, making infants more susceptible to other infections and illnesses [127].

Antiretroviral therapy during pregnancy, particularly containing zidovudine, has been associated with adverse perinatal outcomes, potentially due to mitochondrial toxicity, oxidative stress, and apoptosis in the placenta [128]. Antiretrovirals have also been linked to hepatotoxicity, cardiotoxicity, and nephrotoxicity, potentially through oxidative stress mechanisms [129]. However, antiviral medications for influenza during pregnancy were not associated with increased rates of preterm birth, premature rupture of membranes, or other adverse outcomes in mothers or neonates [130]. For severe neonatal viral infections like herpes simplex virus and cytomegalovirus, nucleoside analogs remain the primary treatment, but they can cause toxicity and drug resistance. There is a concerning lack of new antivirals in development for potentially fatal neonatal viral infections [131]. Neonatal glucocorticoid therapy is effective for treating chronic lung disease in premature infants but can lead to long-term cardiovascular effects due to oxidative stress and reduced nitric oxide bioavailability. Interestingly, combined treatment with antioxidant vitamins prevented these adverse effects, suggesting a potential protective role for antioxidants in neonatal antiviral therapy [132]. This has significant implications for drug development, where the goal is to balance antiviral efficacy by minimizing oxidative stress to reduce adverse outcomes in newborns. To that, it is necessary to assess how antivirals affect mitochondrial function, ROS production, and placental health.

Table 2. Oxidative stress biomarkers.

Biomarker	Type of Oxidative Damage	Significance	Sample Type	References
Isoprostanes	Lipid peroxidation	Reflects oxidative damage to lipids; used as a marker of oxidative stress in preterm infants	Cord blood, urine, saliva	[133,134]
Advanced Oxidation Protein Products (AOPP)	Protein oxidation	Measures protein damage caused by ROS; associated with various neonatal diseases	Cord blood, plasma, saliva	[133,135]
Non-Protein-Bound Iron (NPBI)	Iron-mediated oxidative damage	Indicator of free iron availability, which can catalyze ROS production, linked to oxidative damage	Cord blood, urine	[133]
8-Hydroxy-2′-deoxyguanosine (8-OHdG)	DNA oxidation	Marker of oxidative DNA damage; can indicate long-term risks like cancer or neurodevelopmental disorders	Urine, blood	[136]
Malondialdehyde (MDA)	Lipid peroxidation	Byproduct of lipid peroxidation, associated with cellular damage and oxidative stress in newborns	Cord blood, plasma, urine	[134]
Glutathione (GSH)/Glutathione Disulfide (GSSG)	Redox balance marker	Reflects the cellular oxidative stress status by measuring the balance between reduced and oxidized glutathione	Blood, cord blood, saliva	[133]
Total Antioxidant Capacity (TAC)	Antioxidant defense capacity	Assesses the body's overall ability to neutralize ROS; useful in determining oxidative stress status	Blood, urine, saliva	[135]

Moreover, oxidative stress plays a significant role in neonatal conditions such as jaundice, respiratory distress syndrome (RDS), and brain injury. In the case of neonatal jaundice, oxidative stress exacerbates bilirubin toxicity by elevating ROS levels, which impair the activity of key enzymes involved in bilirubin metabolism, such as UDP-glucuronosyltransferase, hindering bilirubin conjugation and clearance [137]. This is particularly significant in preterm infants whose antioxidant systems are underdeveloped. Unconjugated bilirubin, in the presence of oxidative stress, can cross the blood–brain barrier, increasing the risk of kernicterus and subsequent neurological complications [138]. In respiratory distress syndrome (RDS), oxidative stress is triggered by oxygen therapy and mechanical ventilation, which leads to excessive ROS production. This damages the alveolar epithelial cells and disrupts surfactant production, exacerbating respiratory failure. Furthermore, oxidative stress-induced inflammation activates cytokine release, which worsens lung injury and increases the risk of bronchopulmonary dysplasia (BPD) in preterm infants [139]. Oxidative stress plays a critical role in neonatal brain injury, including periventricular leukomalacia (PVL) and intraventricular hemorrhage (IVH). ROS cause lipid peroxidation and damage to the immature myelin sheath, impairing neural development. Additionally, oxidative stress activates microglia and astrocytes, leading to neuroinflammation and exacerbating brain damage [140]. This is particularly detrimental in preterm infants, where the antioxidant defenses of the brain are insufficient to counteract ROS.

Optimizing drug dosages, frequency, and delivery methods is crucial to reduce oxidative stress while maintaining effective treatment levels. For example, lowering the dose of drugs with high mitochondrial toxicity or modifying the delivery system to allow controlled release may reduce the oxidative burden on the mother and fetus. A careful risk–benefit analysis is essential, particularly for drugs like ribavirin with teratogenic potential, where risks to fetal health often outweigh the benefits. In such cases, safer alternatives or stricter monitoring should be prioritized to protect fetal development. Long-term monitoring of infants exposed to antivirals in utero is also important. Antioxidants alongside antiviral therapy show promise in reducing oxidative stress, offering a potential strategy to balance effective antiviral use with fetal protection. Understanding oxidative stress's role in neonatal outcomes is key to developing safer therapies, and future treatments should focus on reducing mitochondrial dysfunction and ensuring thorough safety evaluations in pregnant populations.

Table 3. Effects of antiviral drugs on oxidative stress.

Mechanism	Antiviral Class/Example	Effects in Pregnancy	Implications for Newborns
Directly Generate ROS	Zidovudine (NRTI), efavirenz (NNRTI), protease inhibitors	Mitochondrial dysfunction, increased oxidative stress, potential fetal growth restriction	Increased ROS production, potential for low birth weight, and neurodevelopmental risks
Impair Antioxidant Defenses	Integrase inhibitors (dolutegravir, raltegravir), neuraminidase inhibitors (oseltamivir)	Disruption of redox balance, compromised placental antioxidant activity	Reduced antioxidant capacity, higher risk of oxidative stress-related conditions

5. Efficacy and Safety of Specific Antioxidants in Reducing Oxidative Stress During Pregnancy

Several antioxidants have demonstrated potential in reducing oxidative stress-related complications during pregnancy, particularly in individuals undergoing antiviral treatments [56,141]. N-acetylcysteine (NAC) is a potent antioxidant that replenishes intracellular glutathione levels. NAC has been widely studied for its protective effects against drug-induced oxidative stress and has shown promise in preventing preterm birth, improving birth weight, and addressing recurrent pregnancy loss [142,143]. In addition to NAC, supplementation with vitamins C and E—well-known antioxidants that scavenge free

radicals—has been suggested to reduce oxidative stress markers. This, in turn, may help prevent complications such as preeclampsia and IUGR, although results across studies remain inconsistent [144,145]. The findings from large-scale randomized controlled trials, such as vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial), indicate that antioxidant supplementation does not significantly reduce the incidence of preeclampsia or other adverse pregnancy outcomes [146]. For instance, the VIP trial showed that women receiving vitamin C (1000 mg) and vitamin E (400 IU) did not experience a lower rate of preeclampsia compared to those receiving a placebo. In fact, the intervention group exhibited a higher risk of gestational hypertension and adverse neonatal outcomes, such as low birth weight [147,148]. Moreover, systematic reviews and meta-analyses have consistently reported no significant benefits of antioxidant supplementation in preventing preeclampsia, severe preeclampsia, preterm birth, or neonatal death when compared to placebo groups. While meta-analyses indicate that vitamin C supplementation does not significantly reduce the incidence of preeclampsia or other adverse pregnancy outcomes, recent research has explored its role as an adjunctive treatment in managing oxidative stress during COVID-19. Studies have suggested that high-dose vitamin C may help modulate immune responses and reduce inflammation in non-pregnant populations. However, it is important to note that these findings have not yet been studied in pregnant individuals or newborns. This gap underscores the need for further investigation into the safety and efficacy of vitamin C therapy in these vulnerable populations.

This suggests that the anticipated protective effects of antioxidants against oxidative stress during pregnancy may not translate into clinical benefits. Regarding safety, while antioxidants are generally considered safe and have a low incidence of severe adverse effects, the results from clinical trials raise concerns about their use in specific populations. The increased risk of gestational hypertension and adverse neonatal outcomes observed in some studies indicate that antioxidant supplementation may not be without risks. Fabrizio et al. emphasize that despite the theoretical safety of vitamins, the clinical implications of their supplementation during pregnancy warrant caution [149]. The lack of demonstrated efficacy combined with potential risks suggests that healthcare providers should carefully consider the use of antioxidant supplements in pregnant women, particularly those at risk for complications like preeclampsia [7]. Fabrizio et al. also suggest that research should focus on the timing and context of supplementation rather than continuing to pursue trials that have not yielded positive results in the past. Another antioxidant, resveratrol, has been recognized for its potential to improve placental function and fetal growth and mitigate complications associated with gestational diabetes and maternal obesity [150,151]. However, evidence from human studies remains limited and contradictory [152]. Resveratrol intake has been shown to decrease inflammation and oxidative stress in placental and embryonic tissues, which are critical factors in adverse pregnancy outcomes [152,153]. Low doses may provide beneficial effects, while higher doses could potentially lead to adverse outcomes, suggesting the need for careful dosage considerations in supplementation.

Melatonin has shown promising effects in addressing placental insufficiency and related complications. It enhances antioxidant capacity in the placenta by upregulating antioxidant enzymes like thioredoxin, glutamate–cysteine ligase, and manganese SOD [154,155]. Melatonin also reduces oxidative stress by inhibiting NADPH- and iron-dependent lipid peroxidation in placental mitochondria [156]. In undernourished pregnancies, melatonin improves placental efficiency and birth weight [155]. It may reduce soluble fms-like tyrosine kinase-1 secretion from trophoblasts, potentially benefiting preeclampsia management [154]. Pregnancies complicated by placental insufficiency show altered melatonin secretion patterns, with lower systemic and placental concentrations and reduced receptor expression [157]. While small intervention studies suggest melatonin treatment may prolong pregnancy and improve outcomes, large-scale randomized controlled trials are still needed to confirm its efficacy [157].

Similarly, curcumin, the main polyphenol in turmeric, has shown promising effects in animal studies for improving pregnancy outcomes, particularly in complications like

IUGR [158]. Its anti-inflammatory, antioxidant, and antiangiogenic properties present a potential therapeutic agent for various pregnancy-related disorders, including gestational diabetes mellitus, preeclampsia, and fetal growth disorders [159]. Curcumin's pleiotropic functions and safety profile have led to increased interest in its use during pregnancy [160]. Curcumin has demonstrated beneficial effects on various chronic diseases in humans [161]. Nevertheless, research on its impact during human pregnancy remains limited. Current evidence originates from animal models and in vitro studies, highlighting the need for further investigation in human clinical trials to fully understand curcumin's potential benefits and risks in pregnancy [159,160].

Understanding oxidative stress mechanisms during pregnancy and their interactions with antiviral therapies is crucial for developing safer and more effective treatments for pregnant patients. Antiviral drugs can influence placental antioxidant systems by depleting essential antioxidants or disrupting pathways that mitigate oxidative stress (Figure 2). Therefore, selecting antivirals that support placental health and minimize oxidative damage is critical. Several antiviral drugs may have inherent antioxidant properties, providing an opportunity to create synergistic combinations that treat viral infections and reduce oxidative stress. This approach can develop multi-functional therapies that are effective and safe for the mother and fetus.

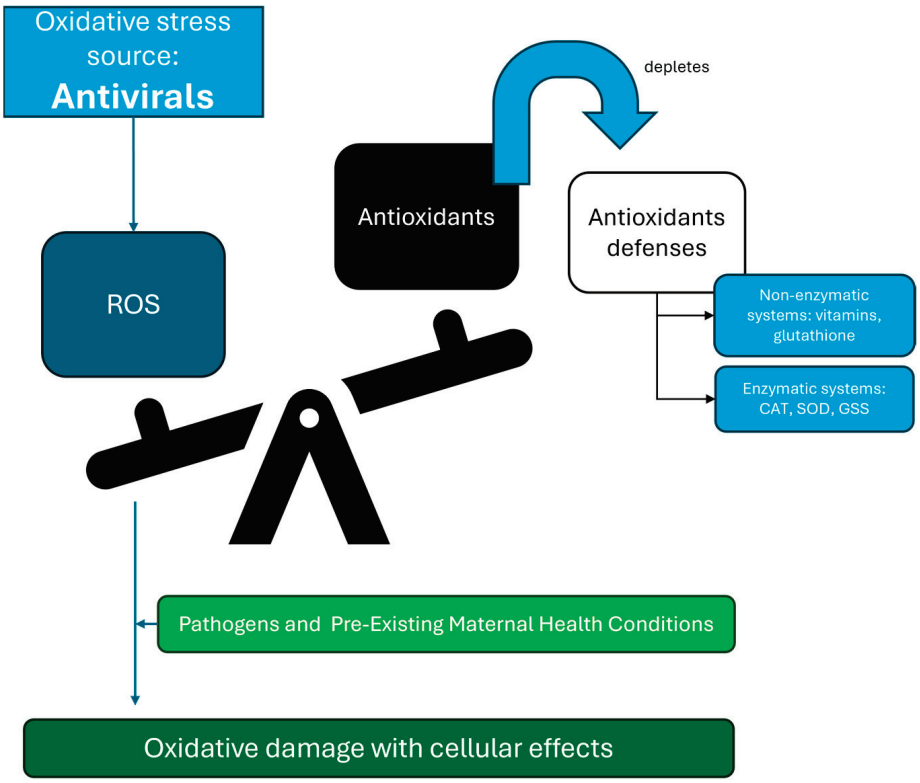


Figure 2. Illustration of the interplay between oxidative stress and antioxidant defenses.

Minimizing the impact of antivirals on fetal oxidative stress is equally important for reducing developmental complications. Certain drugs may cross the placental barrier and affect the fetus's antioxidant defenses. Selecting antivirals with minimal effects on fetal oxidative stress can lower the risk of developmental issues. Implementing targeted drug selection, developing combination therapies including antioxidants, and regularly monitoring oxidative stress markers during antiviral treatment are key strategies to prevent adverse pregnancy outcomes.

To optimize antiviral regimens for pregnant patients, comprehensive research is needed to evaluate the effects of various antivirals on placental and fetal oxidative stress mechanisms. Developing antioxidant-enhanced antivirals and exploring combination

therapies with antioxidants can help mitigate oxidative stress-related risks. Additionally, identifying biomarkers of oxidative stress will guide treatment decisions and therapy effectiveness. Finally, dosing strategies should be optimized to balance antiviral efficacy with minimal oxidative stress, ensuring safer treatments for pregnant patients and their developing infants.

6. Potential Strategies to Mitigate Antiviral-Induced Oxidative Stress

Here, several proposed strategies to advance research on assessing oxidative stress induced by antiviral drugs are discussed. In vulnerable populations, such as pregnant patients, both the viral infection and pregnancy itself can increase oxidative stress. Therefore, antiviral drugs should be carefully selected to avoid adding further oxidative burden. The goal is to ensure that treatment does not exacerbate oxidative stress while effectively managing the viral infection.

Pharmacological agents targeting mitochondrial antioxidant systems and inducing antioxidant enzyme expression could be a promising strategy to mitigate antiviral-induced oxidative stress during pregnancy. Mitochondria-targeted antioxidants, particularly MitoQ and SkQ1, have shown promise in addressing oxidative stress-related conditions, including pregnancy complications [53]. These compounds accumulate in mitochondria, the primary cellular ROS source, providing targeted protection against oxidative damage [162]. They have demonstrated beneficial effects in various animal models and some clinical trials [53]. However, timing of administration is crucial, as MitoQ protects against preeclampsia when given in late gestation but exacerbates the condition when administered early in pregnancy. This occurs due to mild oxidative stress that is necessary for proper placentation [163,164]. Mitochondria-targeted antioxidants act by preventing chain reactions of cardiolipin peroxidation initiated by mitochondrial ROS, which play a key role in many degenerative processes. These types of antioxidants could help preserve placental function and fetal development. Stimulating the expression of antioxidant enzymes like SOD and CAT can enhance the body's natural defense mechanisms against oxidative stress preparing cells to better cope with increased oxidative stress from antiviral therapy, providing sustained protection against various forms of oxidative damage. Combining enzyme inducers with mitochondria-targeted antioxidants could offer comprehensive protection against oxidative stress [165]. Adjunctive therapies like coenzyme Q10 (CoQ10) and alpha-lipoic acid also provide potent antioxidant support, reducing oxidative stress by regenerating other antioxidants and protecting mitochondrial function [166]. Combining these strategies can help mitigate oxidative stress induced by antiviral therapies, thus improving patient outcomes and quality of life. Mechanistic insights into how specific antivirals induce oxidative stress at the cellular level are critical. Such studies can identify which drugs or drug combinations are most likely to increase ROS production, allowing for the design of safer therapeutic regimens or adjunctive antioxidant therapies. Optimizing dosing strategies based on insights into oxidative stress mechanisms is another crucial approach, as it allows for balancing antiviral efficacy while minimizing oxidative harm.

The approach that involves targeting cellular oxidoreductases and the modulation of the Nrf2 pathway might also be beneficial. Cellular oxidoreductases play a crucial role in viral entry and infection-associated oxidative stress, rendering them potential targets for antiviral strategies [167]. Viruses can modulate the Nrf2 pathway, affecting viral replication and disease progression [168]. Studies have shown an inverse relationship between Nrf2 expression and viral entry/replication, with Nrf2 knockdown increasing influenza virus susceptibility and Nrf2 activation via compounds like sulforaphane and epigallocatechin gallate decreasing viral entry and replication [169]. These findings suggest that targeting oxidoreductases with inhibitors and modulating the Nrf2 pathway could be promising approaches for suppressing viral replication and oxidative damage [167,170]. However, careful assessment of benefits and risks is necessary when considering these antiviral strategies [167]. Regular assessment of oxidative stress markers and antioxidant levels can help identify early signs of oxidative damage, enabling timely interventions to minimize

complications. The development of oxidative stress biomarkers is key to personalized medicine in pregnant patients receiving antiviral therapy. Real-time monitoring of oxidative stress levels can guide dose adjustments and reduce the risk of oxidative damage. Additionally, combining antivirals with antioxidant supplements can further reduce the oxidative burden, ensuring safer antiviral regimens [171]. Ongoing clinical trials that include oxidative stress measurements, as well as longitudinal studies tracking children exposed to these drugs in utero, can provide valuable insights into the long-term impact of oxidative stress, guiding future treatment protocols.

Advancements in the study of viral infections, preeclampsia, and IUGR have identified oxidative stress as a shared underlying mechanism driving these conditions. Recent approaches to treating preeclampsia and IUGR have focused on addressing oxidative stress. In the context of viral infections, these may help mitigate the oxidative damage caused by both viral infections and antiviral treatments during pregnancy. Developing antivirals that minimize oxidative stress could potentially reduce the risk of preeclampsia and IUGR in infected pregnant individuals [172].

Nutrigenetic studies are exploring how genetic variations affect an individual's response to nutrients and their susceptibility to oxidative stress. In the context of viral infections and pregnancy complications, we can have: (1) Personalized nutrition: tailoring dietary interventions based on genetic profiles could help pregnant individuals better manage oxidative stress induced by viral infections. (2) Antioxidant supplementation: identifying genetic markers that predict responsiveness to antioxidant supplementation could help prevent preeclampsia and IUGR in virus-infected pregnant individuals [173].

Recent research into new preventive drugs for preeclampsia and IUGR highlights potential connections to viral infections. Therapeutics that activate the Nrf2 pathway, a key regulator of the antioxidative response, hold promise for counteracting virus-induced oxidative stress and reducing the risk of pregnancy complications. Additionally, mitochondrial-targeted therapies may offer another avenue for intervention, as viruses such as SARS-CoV-2 are known to disrupt mitochondrial function. By preserving mitochondrial health, these therapies could help mitigate oxidative stress-related complications during pregnancy [174].

Future research should focus on investigating the long-term effects of maternal viral infections on placental function and fetal development, developing antiviral treatments that minimize oxidative stress during pregnancy, and exploring the potential of personalized medicine approaches in managing viral infections and preventing related pregnancy complications. Moreover, in vitro studies on oxidative stress and antiviral medications can provide valuable insights that inform the development and refinement of DILIsym X (DSX) software. DILIsym is a computational tool used to predict drug-induced liver injury (DILI) and other adverse drug reactions. The use of DILIsym software can further benefit research by integrating in vitro data on oxidative stress and antiviral medications [175]. In vitro studies provide mechanistic insights into oxidative stress pathways, helping researchers understand how antiviral drugs interact with cellular components and antioxidant defenses [75]. In DILIsym, several in vitro input values can help simulate the interactions and effects of drugs on liver function. Some key in vitro measurements that can be incorporated into DILIsym include Mitochondrial Function (mitochondrial membrane potential, ATP levels, ROS production, mitochondrial mass), Cell Health Indicators (cell viability, apoptosis and necrosis markers, cellular ATP content), Oxidative Stress Markers (GSH levels, ROS levels, Lipid peroxidation), Enzyme and Transporter Activity (bile acid transporter inhibition (e.g., BSEP, MRP3/4), enzyme inhibition (e.g., UGT1A1)), Biomarkers (traditional liver injury biomarkers (e.g., ALT, AST), novel biomarkers (e.g., HMGB1, K18)), Metabolic Parameters (metabolic rate of the drug, formation of reactive metabolites). These in vitro measurements help DILIsym simulate the dose–response relationship and predict potential hepatotoxicity in vivo [176]. This information is vital for simulating drug effects in DILIsym, especially in defining dose–response relationships and identifying threshold levels at which oxidative stress becomes significant. In vitro experiments are crucial to

identifying reliable oxidative stress biomarkers, which can be integrated into DILIsym to enhance predictive accuracy and ensure safer drug regimens. These experiments help determine the dose–response relationship between antiviral drug concentrations and oxidative stress markers, essential for accurate DILIsym simulation. Identifying threshold levels of oxidative stress allows for the establishment of safety margins within the software [177].

Integrating physiologically based pharmacokinetic (PBPK) models, such as those in GastroPlus and PK-Sim, is crucial for simulating drug exposure during pregnancy [178]. These models predict antiviral drug interactions within the complex physiological environment of pregnancy, aiding in the optimization of dosing strategies to balance viral suppression and minimize oxidative stress-related complications. In vitro research also maps the metabolic pathways of antiviral drugs, including the generation of reactive metabolites that contribute to oxidative stress [179]. Studying drug interactions at the cellular level can predict potential adverse effects from concurrent medication use. Additionally, in silico drug combination assays using GastroPlus or PK-Sim can simulate drug–drug interactions, further optimizing therapy [180,181]. Researchers can use in vitro findings to test various scenarios in DILIsym, such as different dosing regimens or patient populations, to predict outcomes more accurately.

7. Conclusions

The literature indicates that antiretrovirals, particularly zidovudine, can cause mitochondrial dysfunction, a major source of oxidative stress. This dysfunction may impair energy production and lead to the accumulation of ROS. In the placenta, oxidative stress can compromise function and lead to complications such as preeclampsia, IUGR, and other adverse outcomes. Oxidative stress may also impact fetal brain development, with animal studies showing impaired cognitive functions following prenatal exposure to antiretrovirals. Furthermore, oxidative stress is linked to conditions like small gestational age and low birth weight, critical indicators of fetal health. Increased oxidative stress has also been associated with preterm labor, contributing to complications like respiratory distress and long-term developmental issues in newborns. Antiretrovirals are crucial for managing HIV during pregnancy, but ARV also poses risks related to oxidative stress. Studies on the oxidative effects of antiretrovirals provide insight into how specific drugs and dosages lead to increased ROS production and mitochondrial dysfunction. Understanding these mechanisms is essential for identifying which drugs or combinations are most likely to induce oxidative stress. By pinpointing the pathways through which antiretrovirals cause oxidative stress, researchers can develop therapies that either avoid these pathways or incorporate adjunctive treatments, such as antioxidants, to counteract these effects. Insights gained from oxidative stress studies can be integrated into clinical guidelines, helping healthcare providers select the safest and most effective ART regimens for pregnant women. This could include specific recommendations on drug choices, dosage adjustments, and monitoring protocols. These guidelines can also include risk management strategies, such as more frequent monitoring of oxidative stress markers in women on high-risk regimens or adjusting therapy based on oxidative stress levels.

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Review

Caffeine: The Story beyond Oxygen-Induced Lung and Brain Injury in Neonatal Animal Models—A Narrative Review

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Abstract: Caffeine is one of the most commonly used drugs in intensive care to stimulate the respiratory control mechanisms of very preterm infants. Respiratory instability, due to the degree of immaturity at birth, results in apnea of prematurity (AOP), hyperoxic, hypoxic, and intermittent hypoxic episodes. Oxidative stress cannot be avoided as a direct reaction and leads to neurological developmental deficits and even a higher prevalence of respiratory diseases in the further development of premature infants. Due to the proven antioxidant effect of caffeine in early use, largely protective effects on clinical outcomes can be observed. This is also impressively observed in experimental studies of caffeine application in oxidative stress-adapted rodent models of damage to the developing brain and lungs. However, caffeine shows undesirable effects outside these oxygen toxicity injury models. This review shows the effects of caffeine in hyperoxic, hypoxic/hypoxic-ischemic, and intermittent hypoxic rodent injury models, but also the negative effects on the rodent organism when caffeine is administered without exogenous oxidative stress. The narrative analysis of caffeine benefits in cerebral and pulmonary preterm infant models supports protective caffeine use but should be given critical consideration when considering caffeine treatment beyond the recommended corrected gestational age.

Keywords: hyperoxia; hypoxia; hypoxia–ischemia; intermittent hypoxia; oxidative stress; caffeine; newborn rodent; developing brain; immature lung

1. Introduction

1.1. Caffeine in Preterm Infants

The use of caffeine in premature infants is undisputed. In the late 1970s, Aranda et al. [1] already described the reduction of AOP frequency in preterm infants aged 28 weeks on average. The physiologically influenced effects of caffeine are due to the stimulation of the respiratory control centers in the medulla by competitive inhibition of adenosine and increased contractility of the diaphragm [2,3]. The fact that caffeine is still the first drug of choice for the treatment of AOP is justified by a large number of clinical studies with a high level of evidence, beginning with the CAP studies by Schmidt et al. [4] on the proven benefits of caffeine in the treatment of premature infants [5]. Besides reduced apnea frequency, the most important caffeine effects are reduced incidences of BPD, chronic lung disease, death, and cerebral palsy [4,6–9]. By adolescence, the caffeine effect is lost in preterm infants, but caffeine-treated children still benefit from improvements in motor function and visual perception at the ages of 5 and 11 years [10–13]. With lower levels of evidence, caffeine appears to reduce postoperative apnea and induce anti-inflammatory cytokine profiles [14]. An ongoing debate on the timing of the initiation of caffeine therapy for preterm infants seems to confirm an early start within the first three days of life [15–17]. Individual clinical studies repeatedly report the harmful effects of caffeine in premature newborns. These include reduced cerebral oxygenation and decreased cerebral and intestinal blood flow velocity [18,19], increased blood pressure and tachycardia [20], a pro-inflammatory cytokine profile [21], as well as an increase in seizure frequency and seizure burden [22].

In the current context, Parladori et al. [23] revealed an improvement in cerebrovascular reactivity and pointed to the safety of caffeine in a hemodynamically vulnerable phase with positive effects on cerebral hemodynamics.

1.2. Pharmacology of Caffeine

Caffeine is hydrophilic and lipid-soluble, can therefore be easily distributed in all tissues and body fluids, and is membrane-permeable [3]. However, there are differences between the metabolism of newborns and adults when caffeine is catalyzed in the liver. The reason for this is the immature enzyme production of CYP1A2 in the liver of newborn and premature infants. CYP1A2, also known as cytochrome P450, catalyzes caffeine primarily into paraxanthine, theobromine, and theophylline [24]. During the initial months of life, caffeine is excreted renally, in an almost unmetabolized form, perhaps due to lower, ontogeny-related CYP1A2 activity. As a result, the metabolic products tend to play a subordinate role in potential caffeine effects until the newborn metabolism is fully available at approximately one year of age. This is also accompanied by a slower metabolism and longer serum half-life of caffeine [3,25–27]. These processes can be influenced by the immaturity of the organism, body weight, and necessary nutritional strategies [28–30].

The pharmacological effects of caffeine in clinically relevant doses for the premature infant are mediated, on the one hand, via non-specific antagonism at the adenosine receptor (AdoR) subtypes AdoRA1, AdoRA2a, and AdoRA2b receptors [31–35]. Here, genetic variability due to polymorphisms is not negligible and can modulate the effects of caffeine [36]. On the other hand, experimental studies also point to an important role of caffeine via antioxidant properties [37–40], with high relevance to the fact that premature infants have an immature antioxidant enzyme system [41]. This is accompanied by anti-inflammatory and cell death inhibitory effects, with communication via relevant cellular signaling pathways playing a more important role in mediating the protective effects of caffeine. This aspect is presented in the discussion based on the data collected in this review article.

1.3. Postnatal Oxidative Stress Injury Models for the Immature Lung and Developing Brain

Oxidative stress, as a fundamental risk factor for initially induced diseases of prematurity, is not new and is referred to as oxygen-related diseases of prematurity [42]. Premature infants are highly vulnerable to oxidative stress, which is already unavoidable due to premature birth [43,44]. Early gestational age and low birth weight are negatively correlated with an adequate cellular response to ROS [45], which can be increased by respiratory instability and oxygen therapies being initiated [42,46]. An essential tool for managing the consequences of oxygen adaptation and thus oxidative stress is to observe the oxygen saturation of the premature infant and make sensitive adjustments as required [47]. Despite reliable findings on therapy-optimized oxygen saturations, further studies with reliable outcome results are still required.

Looking at the pulmonary development and the maturation of the brain, which is orchestrated in the intrauterine environment under hypoxic conditions, it is comprehensible and provable that a too-early change due to a too-early birth into an extrauterine hyperoxic environment is associated with oxidative stress. Especially in the last trimester of pregnancy, neuronal processes such as cell division, differentiation, migration, synaptogenesis, myelination, plasticity, and physiological cell death are very vulnerable to non-physiological noxae. The same applies to pulmonary development, which, in prematurely born children, must first develop from the saccular phase into the alveolar mature phase. Oxidative stress causes impairments in the structure and, thus, in the function of both immature organ systems due to multiple mechanisms [48,49]. Some of these causes consequential damage into adulthood. This may increase the risk of adults who survive a premature birth developing chronic diseases, including respiratory disorders such as asthma or COPD, as well as neurological and psychiatric disorders such as ADHD or autism spectrum disorder [50,51]. It should be noted that the more immature newborns are the more susceptible they are to redox-sensitive situations. The risk of developing short- and long-term neurological com-

plications is not only based on the consequences of oxidative stress on the premature brain. It is now known that children with severe BPD, in addition to pulmonary dysfunction in adolescence, can also be neurologically impaired in the long term without additional brain injury [52–54].

1.3.1. Oxidative Stress in Premature

There are various scenarios in which oxidative stress can arise and have an impact on a premature situation. Hyperoxia (HY) per se is unavoidable. The change from the hypoxic environment of the fetus (25–30 mmHg), which is also necessary for normal development, to a hyperoxic environment during birth (75–85 mmHg), generates relative hyperoxia [55], which leads to oxidative stress in an underdeveloped antioxidant system and a lack of alveolar surfactant. Respiratory instabilities sometimes require medically induced oxygen supplementation, which then leads to absolute hyperoxia. In addition, inadequate respiratory control, with apneas and impaired inspiratory efforts, are major triggers for intermittent hypoxia (IH) events [56]. There are few data on the possible long-term consequences of postnatal IH. Experimental and clinical data suggest the induction of pathological cascades via an oxidative stress-induced signaling pathway, with subsequent inflammation [57,58]. Besides asphyxia, hypoxia (HO) or hypoxia/ischemia (HI) in the vulnerable phase of premature infants is one of the most common causes of death and morbidity [59,60]. There is a generalized higher risk of hypoxia events the more immature the organism is at birth, and this is compounded by fetal immobilization with slowed metabolism and reduced respiratory drive during the transition from hypoxic to hyperoxic environments [60]. Hypoxia and hypoxia/ischemia cause oxidative stress and are, in turn, involved in the pathogenesis of neurological diseases such as hypoxic–ischemic encephalopathy [61]. Neuroinflammation and mitochondrial dysfunction alter the cellular status, resulting in irreversible damage [46,62].

1.3.2. Postnatal Oxidative Stress Injury Models

The adequate injury models used for experimental research are based on the transferred clinical oxygen conditions from the neonatal situation. The rodent hyperoxia (HY) injury model is largely based on FiO_2 80% oxygen, which is clinically avoided in neonatal situations. The natural fetal situation during the last trimester of pregnancy is physiologically consistent with a low oxygen concentration until parturition in room air causes an increase in oxygen partial pressure from 25–30 mmHg to 75–85 mmHg [60]. The exogenous rodent hyperoxia model provides a 4-fold increase in oxygen levels and causes an increase in in vivo oxygen partial pressure of 55–165 mmHg in newborn mice [63]. Therefore, 80% oxygen exposure mimics a large increase in oxygen concentration in a neonate with a very immature stage of brain and lung development. The plausibility of the model is based on demonstrably comparable neurological and pulmonary pathologies in premature infants with extremely low and very low birth weights [38,39,48,63–75].

Hypoxia (HO), with oxygen as the only variable, tends to be a criterion for reaching the pathological state in respiratory-associated rodent models. In experimental hypoxic studies in neonatology research, hypoxia is used to represent oxygen deficiency per se [76–78]. Triggering factors in the clinical situation for hypoxia or transient hypoxia are respiratory instabilities, e.g., apnea, infections of the upper respiratory tract, as well as hypoxia under hyperoxia during ventilation of the immature premature infant. Chronic hypoxia in newborn rodents often serves as a model of BPD-induced brain damage [79] and is simulated with oxygen concentrations of usually 8–10% oxygen, whereby the duration of exposure is highly variable. In the case of alternating oxygen concentrations in FiO_2 between normoxia (21% O_2) and hypoxia, the term intermittent hypoxia (IH) is used. In the neonatal clinical setting, the PaO_2 drops to at least 80% [58]. This is also caused by underdeveloped respiratory control and limited lung capacity, which can be exacerbated by BPD. The experimental models are quite variable, with intermittent oxygen concentrations between normoxia and hypoxia (21%/ 5% O_2) or moderate hyperoxia with hypoxia (50%/5–12% O_2) [80–82].

Hypoxia and intermittent hypoxia-injury models usually cause high variability. Using the Rice–Vannuccie model [83,84], i.e., hypoxia after an ischemic insult (HI), this can be manifested. The model is based on a unilateral ligation of the carotid artery with subsequent recovery of the pups under the care of the dam (~1–2 h), only to be exposed to hypoxia. The pups remain under hypoxia (8–10% O_2) for 1–3 h. Subsequent comparisons are made between the damaged hemisphere and the contralateral hemisphere exposed to hypoxia without ischemia. Clinically, this model represents perinatal asphyxia and hypoxic–ischemic encephalopathy (HIE).

All of these damage models related to oxidative stress reflect different settings in the everyday clinical care of perinatal centers. However, they all have one thing in common: a wide range of neuronal and pulmonary damage occurs depending on the severity, duration, and organ-related developmental time of the damage. Even very short phases of oxidative stress cause degeneration, impaired proliferation, or differentiation of precursor cells. Ultimately, these impairments can essentially disrupt function and thus cause a poor long-term outcome [48,67,72,85–89].

This review focuses on rodent oxygen damage models due to the large amount of experimental data available. It should not go unmentioned that large animal models, such as the premature pig or sheep, can extend neuronal and pulmonary development as a premature infant model for developmental studies or investigations of other variables [90,91].

1.3.3. Rodent Model for Preterm Brain Development

In neuroscience, the development of differences between humans and the maturation of the rodent brain is well known. The newborn rodent is very well suited as a model organism for the comparative neuronal development of premature infants [92,93]. Pups are often used within the first two weeks of life. This is due to the phase of rapid brain growth [94]. This phase in rodents is comparable to the developmental stage of premature infants in the 23rd week of pregnancy. At this age, the most important steps of brain development can be deciphered, which occur in both rodents and humans, and thus describe a comparable time course of postnatal brain development in all species [93,95].

1.3.4. Rodent Model for Postnatal Pulmonal Maturation

Oxygen toxicity-induced pulmonary injury models [66,96] are comparable in pathology to human lung-injured preterm infants [97–99]. In newborn postnatal rats, the respective developmental stage at birth correlates with the saccular phase (E18–P4; human gestational age 24–38 weeks) and with the first phase of alveolarization (P4–P21; human gestational age 36 weeks to postnatal age), which overlaps with the second/continuous phase of alveolarization (P14–P60) [97,100]. Following these developmental dependencies, rodents are usually used in oxygen-related models from the day of birth until about the third week of life.

1.4. Objective of This Study

The aim of this review is to provide a comprehensive overview of the effects of caffeine in the recognized postnatal oxygen toxicity models and to specifically outline the effects of caffeine in non-oxygen toxicity models. This narrative analysis is intended to provide the basis for discussion of whether the benefits of caffeine in cerebral and pulmonary preterm neonatal injury models warrant caffeine treatment in the clinical setting of preterm neonatal care beyond the recommended corrected gestational age of 34 weeks gestation (postmenstrual age, PMA).

2. Materials and Methods

The work included a descriptive study with a narrative review to demonstrate caffeine effects in the associated preterm oxidative stress rodent injury models of the immature lung and developing brain. A major part of the review describes caffeine effects in the absence of oxygen toxicity in the pups.

A structured search of PubMed, Scopus, and Google Scholar databases was performed within the last 20 years (2004–2024) using descriptors such as Medical Subjects Headings (MeSH) as well as entry terms to explicitly examine the title, abstract, and study design in the methods section. Likewise, reference lists were used to further search for related references in the selected articles. Individual images from BioRender.com were used to create the overview image and the graphic additions in the tables.

3. Results

3.1. Caffeine and the Hyperoxic-Injury Model

3.1.1. Caffeine Protects the Immature Lungs Affected by Hyperoxia

To compare the data recorded for the use of caffeine in postnatal rodent pulmonary injury models by high oxygen concentrations (HY), eight studies [101–108] were included in this review (Table 1). The rodent species used included mice (C57Bl/6, FVB/n), rats (Sprague Dawley), and rabbits (New Zealand white).

Table 1. Caffeine effects in pulmonary hyperoxia-induced damage model.

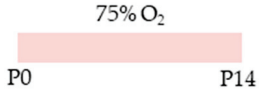
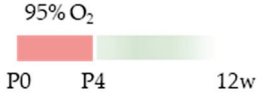
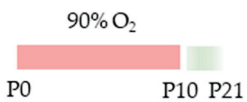
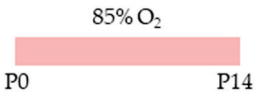
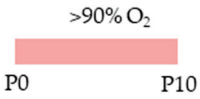
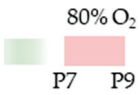
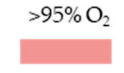
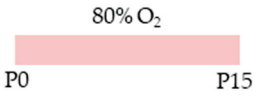
Species	Oxygen Exposure	Caffeine	Caffeine and Pulmonary Hyperoxia		Ref.
					
 C57Bl/6 mice	 75% O_2 P0 P14	1 g/L lactate dam P0–P14	**Pulmonary protection** antioxidative anti-apoptotic anti-inflammatory reduced lung architecture simplification increased surfactant protein	**Pulmonary side effects** modulated NfκB signaling pathway modulated AdoRA2a expression **Side effects** weight gain	[101]
 C57Bl/6 mice	 95% O_2 P0 P4 12w	20 mg/kg i.p. P0–P4	**Pulmonary protection** improved microvascularization (male) reduced lung architecture simplification	**Pulmonary side effects** modulated HIF signaling pathway **Side effects** weight gain	[102]
 SD rat	 90% O_2 P0 P10 P21	20 mg/kg i.p. P2–P10/P21	**Pulmonary protection** antioxidative anti-apoptotic improved microvascularization reduced lung architecture simplification reduced endoplasmic (ER) stress reduced mitochondrial dysfunction		[103]

Table 1. *Cont.*

Species	Oxygen Exposure	Caffeine	Caffeine and Pulmonary Hyperoxia		Ref.
					
 C57Bl/6 mice	 85% O_2 P0 P14	25 mg/kg i.p. P0–P13	**Pulmonary protection** none	**Pulmonary side effects** increased phosphorylation Smad2 **Side effects** weight gain	[104]
 SD rat	 >90% O_2 P0 P10	20 mg/kg i.p. P2–P21	**Pulmonary protection** improved microvascularization reduced lung architecture simplification	**Pulmonary side effects** reduced cAMP level **Side effects** reduced mortality weight gain	[105]
 SD rat	 80% O_2 P7 P9	10 mg/kg i.p. P7	**Pulmonary protection** antioxidant anti-apoptotic anti-inflammatory reduced lung architecture simplification		[106]
 New Zealand white rabbit	 >95% O_2 E29/P0 P5	10 mg/kg i.p. P0 5 mg/kg i.p. P1–P5	**Pulmonary protection** anti-inflammatory improved lung function reduced lung architecture simplification		[107]
 FVB/n mice	 80% O_2 P0 P15	20 mg/kg i.p. P1 10 mg/kg i.p. P2–P15	**Pulmonary protection** none	**Pulmonary damage** pro-apoptotic pro-inflammatory exacerbated lung architecture reduced AECII counts **Pulmonary side effects** reduced AdoRA2a expression	[108]

The colors in the schematic experimental designs indicate hyperoxia (red) with increasing intensity to inspiratory oxygen concentration and exposure to room air (21% O_2, green).

The beginning of hyperoxia in these studies primarily starts on the day of birth (P0), except for in the study by Sadek et al. [106], which starts on postnatal day 7 (P7). The caffeine concentrations used relate to caffeine base and range from 5 to 25 mg/kg/day, whereby caffeine was administered constantly over time in the same concentration or starting with a bolus on the first day of administration. In the study by Chen et al. [101], caffeine was administered via the lactating mother. The start of caffeine therapy is either identical to the start of oxygen exposure or scheduled up to 48 h after hyperoxia.

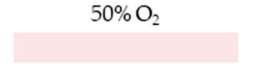
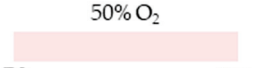
In six [101–103,105–107] of the eight studies, a clear protective effect of caffeine on hyperoxia-damaged lungs was demonstrated. Overall, anti-oxidative, anti-inflammatory, and anti-apoptotic effects were shown, which were accompanied by an improvement in the damaged lung morphology. Some studies considered the signaling pathways involved and were able to demonstrate the modulation of redox-sensitive signaling mediations via NfκB [101] or HIF [102]. Chen et al. [101] also demonstrated increased surfactant translation. Improved microvascularization was demonstrated in the studies by Jing et al. [105], Dumpa et al. [102], and Teng and colleagues [103]. Teng et al. also proved that caffeine reduced ER

stress induced by hyperoxic exposure as well as mitochondrial dysfunction [103]. Two studies were unable to show a protective effect for caffeine after hyperoxia damage [104,108], with Dayanim et al. [108] demonstrating not only pro-apoptotic and pro-inflammatory caffeine effects but also an influence on pulmonary cellular organization. The expression of AdoRA2a was modulated in two out of these eight studies [101,108]. Hyperoxia in addition to caffeine led to a demonstrable weight gain in the pups in four of the studies [101,102,104,105].

3.1.2. Caffeine Protects the Developing Brain Affected by Hyperoxia

To compare the data recorded for the use of caffeine in postnatal rodent brain injury models by high oxygen concentrations (HY), two studies [109,110] were included in this review (Table 2). Rats (Sprague Dawley) were used as the rodent species in these studies.

Table 2. Caffeine effects in neuronal hyperoxia-induced damage model.

Species	Oxygen Exposure	Caffeine	Caffeine and Neuronal Hyperoxia		Ref.
					
 SD rat	 50% O_2 P0 P14	20 mg/kg i.p. P0 5 mg/kg i.p. P1–P14	**Neuroprotection** anti-apoptotic anti-inflammatory protected against demyelination	**Neurodegeneration/side effects** n/a	[109]
 SD rat	 50% O_2 P0 P14	LoC: 20 mg/kg i.p. P0 5 mg/kg i.p. P1–P14 HiC: 80 mg/kg i.p. P0 20 mg/kg i.p. P1–P14	**Neuroprotection** LoC: antioxidant anti-apoptotic reduced neuronal pyknosis increased neurons and dendritogenesis HiC: antioxidant anti-apoptotic increased neurons and dendritogenesis protected from demyelination	**Neurodegeneration/side effects** LoC: reduced AdoRA1a/2a expression weight gain HiC: intraventricular haemorrhages histopathological abnormalities reduced AdoRA1a/2a expression weight loss	[110]

The red color in the schematic experimental designs indicates hyperoxia (red) in the inspiratory oxygen concentration.

Both studies used PaO_2 50% with an exposure duration of 14 days. Caffeine administration started at the same time as hyperoxia on the day of birth, and both studies started at P0 with a bolus of 20 mg/kg/day and 5 mg/kg/day thereafter. Soontarapornchai et al. [110] extended the trials to the clinically adapted dose (LoC) with high-dose caffeine (HiC) of 80 mg/kg/day and 20 mg/kg/day.

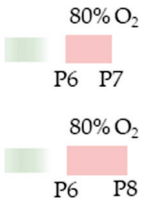
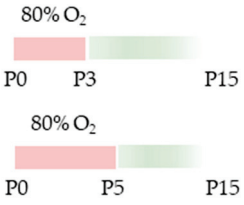
In both studies, caffeine demonstrated its neuroprotective effect via antioxidant, anti-inflammatory, and/or anti-apoptotic effects [109,110]. Batool et al. [109] showed a reduction in demyelination after hyperoxia, as did Soontarapornchai et al. [110] under high caffeine doses. Caffeine mediated more neurons, increased dendritogenesis, and reduced AdoRA1 and AdoRA2a expression at clinically relevant caffeine doses as well as at high concentrations. Adverse effects occurred at high caffeine doses, including IVH and histopathologic abnormalities. Low-dose caffeine led to weight gain under HY, while high-dose caffeine resulted in weight loss [110].

3.1.3. Caffeine Protects the Lung–Brain Axis Affected by Hyperoxia

Very few research groups seem to study the effects of caffeine on the lung–brain axis in parallel. In our group, we have so far been able to study the neuronal as well as pulmonary effects of caffeine in two rat hyperoxia injury models with different experimental designs

(Table 3) [38,39,68–71,75]. The same animal cohort was examined for several aspects of the analysis.

Table 3. Caffeine effects in pulmonary and neuronal hyperoxia-induced damage model.

Species	Oxygen Exposure	Caffeine	Caffeine and Pulmonary-Neuronal Hyperoxia		Ref.
					
 Wistar rat	 80% O_2 P6 P7 80% O_2 P6 P8	10 mg/kg i.p. P6 10 mg/kg i.p. P6	**Pulmonary protection** antioxidant anti-inflammatory reduced lung architecture simplification	**Neuroprotection** antioxidant anti-apoptotic anti-inflammatory protected from tissue remodeling protected from impaired hippocampal neurogenesis **Neuronal side effects** modulated NfκB/Nrf2 signaling pathway	[38,69,75] The studies use the identical cohorts.
 Wistar rat	 80% O_2 P0 P3 P15 80% O_2 P0 P5 P15	10 mg/kg i.p. P0–P2 10 mg/kg i.p. P0–P4	**Pulmonale Protection** antioxidant anti-apoptotic anti-inflammatory **Pulmonary side effects** modulated NfκB/Nrf2 signaling pathway modulated AdoRA1 expression	**Neuroprotection** protected from impaired hippocampal neurogenesis protected from impaired cerebellar neurogenesis and migration rescued neurotrophins	[39,68,70,71] The studies use the identical cohorts.

The colors in the schematic experimental designs indicate hyperoxia (red) in the inspiratory oxygen concentration and exposure to room air (21% O_2, green).

In the first study, the focus was on perinatal brain and lung injury in the presence of oxygen damage (HY) over 24 and 48 h, starting from P6 [38,69,75]. Intraperitoneally, the Wistar rat pups received 10 mg/kg once before the beginning of hyperoxia. In the second study, two periods of exposure to high oxygen were also determined [39,68,70,71]. The newborn Wistar rats were exposed to 80% O_2 for 3 or 5 days from the day of birth and analyzed; additionally, they were supplemented with extended analyses after survival in room air of up to P15. Caffeine at 10 mg/kg was administered from P0, with plasma level monitoring, every two days.

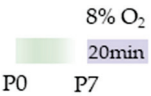
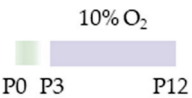
Both studies revealed pulmonary and neuronal protective effects mediated by caffeine. Antioxidant, anti-inflammatory, and anti-apoptotic properties were demonstrated [38,39,68,75]. Caffeine prevented oxygen-mediated simplification of lung structure [75], modulated NfκB/Nrf2 signaling [39], and modulated AdoRA1 expression [68] in lung tissue. Caffeine positively influenced hyperoxia-induced impairment of both hippocampal and cerebellar neurogenesis [38,69–71]. The neurotrophin levels that were reduced by hyperoxia were recovered with caffeine [71].

3.2. Caffeine and the Hypoxic-Injury Model

Caffeine Protects the Developing Brain Affected by Hypoxia

The literature search for experimental studies using only low oxygen concentrations (HO) as oxidative stress triggers revealed two studies with caffeine to be included (Table 4) [111,112]. Both studies conducted neuronal experimental studies with mice, with only Back et al. [112] mentioning the animal species (C57Bl/6).

Table 4. Caffeine effects in neuronal hypoxia-induced damage model.

Species	Oxygen Exposure	Caffeine	Caffeine and Neuronal Hypoxia		Ref.
					
 mice		20 mg/kg p.o. P4 15 mg/kg p.o. P5–P7	**Neuroprotection** reduced HIF1α cortical accumulation	**Neurodegeneration/side effects** n/a	[111]
 C57Bl/6 mice		0.3 g/L lactate dam P2–P12	**Neuroprotection** reduced ventriculomegaly protected against demyelination	**Neurodegeneration/side effects** n/a	[112]

The colors in the schematic experimental designs indicate hypoxia (purple) in the inspiratory oxygen concentration and exposure to room air (21% O_2, green).

In the study by Li et al. [111], seven-day-old mice were exposed to 8% O_2 for 20 min, with caffeine being administered from P4 with a bolus of 20 mg/kg via oral gavage, followed by 15 mg/kg (p.o.) until P7. The mouse pups in the study by Back et al. [112] received caffeine via the lactating dam from P2 for ten days (up to P12) and were exposed to low oxygen concentrations at 10% O_2 from P3 to P12.

The analyzed endpoints of the studies showed a caffeine-prevented HIF1α accumulation in the cortex after hypoxia [111], as well as reduced ventriculomegaly and caffeine-mediated protection against demyelination [112].

3.3. Caffeine and the Hypoxic–Ischemic-Injury Model

Caffeine Protects the Developing Brain Affected by Hypoxia–Ischemia

Nine experimental in vivo studies with induced hypoxia–ischemia (HI) and caffeine application were identified, all of which focused on neuronal effects (Table 5) [113–121].

Table 5. Caffeine effects in neuronal hypoxia–ischemia-induced damage model.

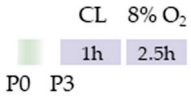
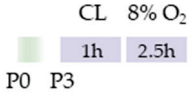
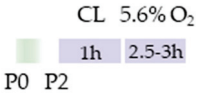
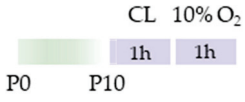
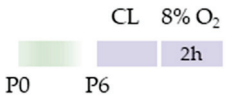
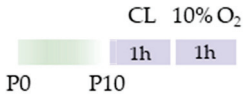
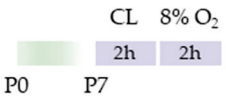
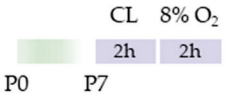
Species	Oxygen Exposure	Caffeine	Caffeine and Neuronal Hypoxia-Ischemia		Ref.
					
 SD rat		10 mg/kg i.p. P2–P6 before HI and post HI	**Neuroprotection** anti-apoptotic	**Neuronal side effects** improved mitochondrial function promoted effects of deacetylation	[113]
 SD rat		10 mg/kg i.p. P2–P6 before HI and post HI	**Neuroprotection** anti-inflammatory restored reduced synapses proteins alleviated the suppressed cognitive impairment reduced ventriculomegaly protected against demyelination promote microglial polarization to the M2 phenotype	**Neurodegeneration/side effects** n/a	[114,115] The studies use the identical cohorts.

Table 5. *Cont.*

Species	Oxygen Exposure	Caffeine	Caffeine and Neuronal Hypoxia-Ischemia		Ref.
					
 SD rat		0.3 g/L lactate dam E8–P16 before HI and P3–P16 post HI	**Neuroprotection** before HI: reduced number of brain infarcts reduced infarct volume restored suppressed EEG brain activity post HI: restored suppressed EEG brain activity	**Neurodegeneration/side effects** n/a	[116]
 C57Bl/6 mice		5 mg/kg i.p. 0 h, 6 h, 12 h, and 24 h post HI	**Neuroprotection** anti-apoptotic anti-inflammatory reduced infarct volume protected against demyelination reduced microglia-activation	**Neurodegeneration/side effects** n/a	[117]
 Wistar rat		10 mg/kg i.p. 0 h, 24 h post HI	**Neuroprotection** improved cognitive and motoric learning (male)	**Neurodegeneration/side effects** n/a	[118]
 C57Bl/6 mice		5 mg/kg i.p. 0 h post HI	**Neuroprotection** improved motoric learning	**Neurodegeneration/side effects** n/a	[119]
 Wistar rat		10 mg/kg i.p. before HI and 0 h, 24 h, 48 h, and 72 h post HI	**Neuroprotection** anti-apoptotic (HC, cortex)	**Neurodegeneration/side effects** n/a	[120]
 Wistar rat		10 mg/kg i.p. 0 h post HI	**Neuroprotection** improved spatial learning (male)	**Neurodegeneration/side effects** n/a	[121]

The colors in the schematic experimental designs indicate the carotid artery ligation (CL) and hypoxia (purple) in the inspiratory oxygen concentration and exposure to room air (21% O_2, green).

Initially, the rats (Wistar, Sprague Dawley) or mice (C57Bl/6) pups were always subjected to carotid artery ligation (CL), although the age varied. Four studies placed the CL closer to birth (P2, P3) [113–116], one study at P6 [118], two studies at P7 [120,121], and two studies at P10 [117,119]. The ischemia set in this way was maintained for from one to two hours, although Potter et al. [118] did not provide any specific details. The subsequent hypoxia after recovery in the mother was performed at 8%, 10%, or 5.6% O_2 and varied between one hour and three hours (see Table 5). In four studies, caffeine was administered directly after hypoxia [117–119,121] and, in some cases, with additional repetitive caffeine doses up to a maximum of 24 h after hypoxia [117,118]. Kilicdag et al. [120] administered the first dose of caffeine before hypoxia and continued the applications until 72 h after

hypoxia. In three studies, rat pups were treated with caffeine prior to CL, starting 24 h before the insult. Caffeine was administered well beyond HI until P6 [113–115]. Sun et al. [116] chose fetal premedication via the pregnant mother from embryonic day 8 (E8) before HI or starting from P3 after HI and continued caffeine treatment via the lactating mother until P16. The caffeine doses for eight of the studies mentioned were from 5 to 10 mg/kg (i.p.). There were five studies that demonstrated that caffeine had anti-oxidative, anti-inflammatory, and/or anti-apoptotic effects [113–115,117,120].

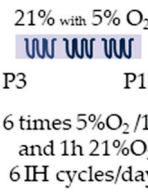
Hypoxia–ischemia led to impairments in cognitive and motor learning. Caffeine was able to protectively influence these deficits in the HI damage model [118,119,121]. The caffeine-mediated effects were somewhat more complex in the studies by Yang et al. [114,115] and Sun et al. [116]. Preconditioning with caffeine before the actual HI insult was able to restore the reduced synaptic proteins, alleviate the suppressed cognitive impairment, reduce ventriculomegaly, protect against demyelination, and promote the polarization of microglia to the M2 phenotype [114,115]. The fetal preconditioned rat pups in the study by Sun et al. [116] displayed a lower number of cerebral infarcts and a lower infarct volume and restored the suppressed EEG brain activity. Application of caffeine only after the HI insult showed no influence on the infarction itself, but positively influenced the suppressed EEG brain activity. A one-day caffeine premedication promoted mitochondrial functions [113].

3.4. Caffeine and the Intermittent-Hypoxic Injury Model

3.4.1. Caffeine Protects the Immature Lung Affected by Intermittent Hypoxia

The search for pulmonary-related experimental studies with intermittent oxygen concentration (IH) as a trigger for oxidative stress resulted in two studies that considered the insult of caffeine (Table 6), both of which originated from the same working group around Jiang et al. [122,123].

Table 6. Caffeine effects in pulmonal intermittent hypoxia-induced damage model.

Species	Oxygen Exposure	Caffeine	Caffeine and Pulmonal Intermittent Hypoxia		Ref.
					
 SD rat		15 mg/kg p.o. P3–P15 * 10 mg/kg i.p. P3–P15 **	**Pulmonary protection** reduced frequency of apnea	**Pulmonary side effects** n/a	[122] * [123] **

The colors in the schematic experimental designs show the hypoxia (purple) in the inspiratory oxygen concentration, with waves to represent the intermittent oxygen concentrations. * The indicated concentration refers to [122]. ** The indicated concentration refers to [123].

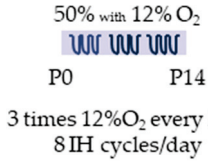
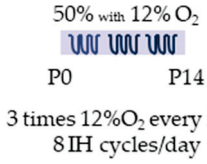
Three-day-old rats (Sprague Dawley) were used in both studies. The study design was identical, but the caffeine doses and the administration route varied. Caffeine was administered either orally at 15 mg/kg [122] or intraperitoneally at 10 mg/kg [123]. Pups were transferred to intermittent hypoxia on postnatal day 3 and remained there until P12. A ten-minute cycle involving an intermittent change from 21% O_2 to 5% O_2 was repeated six times within one hour, followed by a one-hour break below 21% O_2. A total of six IH cycles were performed per day.

The two analyzed caffeine concentrations via different application routes were able to successfully reduce the apnea frequencies triggered by IH per day.

3.4.2. Caffeine Protects the Developing Brain Affected by Intermittent Hypoxia

Intermittent hypoxia (IH) and caffeine as an entrained substance were analyzed in two studies investigating the neuronal effects (Table 7) [109,110].

Table 7. Caffeine effects in neuronal intermittent hypoxia-induced damage model.

Species	Oxygen Exposure	Caffeine	Caffeine and Neuronal Intermittent Hypoxia		Ref.
 SD rat	 50% with 12% O_2 P0 P14 3 times 12%O_2 every 2.4h 8 IH cycles/day	20 mg/kg i.p. P0 5 mg/kg i.p. P1–P14	**Neuroprotection** anti-inflammatory reduced histopathological abnormalities	**Neuronal side effects** hypermyelination **Side effects** reduced enhanced brain/body weight ratio	[109]
 SD rat	 50% with 12% O_2 P0 P14 3 times 12%O_2 every 2.4h 8 IH cycles/day	LoC: 20 mg/kg i.p. P0 5 mg/kg i.p. P1–P14 HiC: 80 mg/kg i.p. P0 20 mg/kg i.p. P1–P14	**Neuroprotection/side effects** LoC: anti-apoptotic increased neurons and dendritogenesis HiC: antioxidant anti-apoptotic increased neurons and dendritogenesis	**Neurodegeneration/side effects** LoC: pro-oxidant reinforced demyelination histopathological abnormalities Neuronal side effects LoC: reduced AdoRA1a expression induced AdoRA2a expression HiC: reduced AdoRA1a expression Side effects LoC: induced body length HiC: reduced brain weight weight loss	[110]

The color in the schematic experimental designs shows the hypoxia (purple) in the inspiratory oxygen concentration, with waves to represent the intermittent oxygen concentrations.

In both studies, newborn rat pups were used for the experiments, with a similar experimental design. Newborn rats (P0, Sprague Dawley) were transferred to IH shortly after birth and remained there until the end of the second week of life (P14). Pups were maintained under moderate hyperoxia (50% O_2) and underwent three episodes of intermittent hypoxia (12% O_2), each lasting 10 min every 2.5 h. A total of eight IH cycles were performed per day. Batool et al. [109] also started the administration of caffeine with a bolus of 20 mg/kg/day at the beginning of IH, followed by 5 mg/kg/day. Soontarapornchai et al. [110] extended the trials to the clinically adapted dose (LoC, 20 mg/kg bolus, 5 mg/kg i.p.) with a high caffeine dose (HiC) of 80 mg/kg/day (bolus) and 20 mg/kg/day (i.p.).

Batool et al. [109] reported an anti-inflammatory effect of caffeine in the IH model of the postnatal rat, which was associated with attenuation of histopathological changes in the cerebral cortex. Side effects in this study were hypermyelination of the juvenile brain, as well as an increased brain–body weight ratio. Examining the effects of caffeine in the study by Soontarapornchai et al. [110], low-dose caffeine (LoC) under IH was found to have anti-apoptotic effects associated with increased neuron numbers as well as increased dendritogenesis. Similarly, caffeine under oxidative stress had a pro-oxidant effect, increased demyelination, and led to histopathological abnormalities in the cerebral

cortex. Higher concentrations (HiC) of caffeine also had an anti-apoptotic effect with increased neurons and dendritogenesis, but they also exhibited an antioxidant effect. Both concentrations were able to modulate AdoRA1a and AdoRA2a gene expression. Low caffeine dosages induced body length, while high dosages led to weight loss as well as reduced brain weight.

3.5. Caffeine and the Immature Lung and the Developing Brain

3.5.1. Caffeine in the Immature Lung without Oxidative Stress

Eight studies [101–105,108,122,124] were included in the analyses of the effect of caffeine on the postnatal immature lung (Table 8). The rodent species used included mice (C57Bl/6, FVB/n) and rats (Sprague Dawley).

Table 8. Caffeine effects in the immature lung under standard conditions.

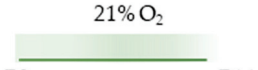
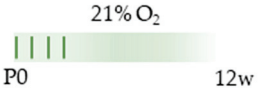
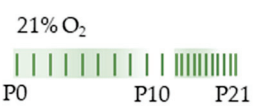
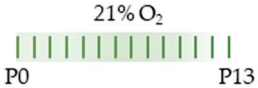
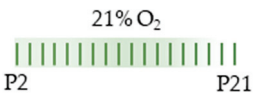
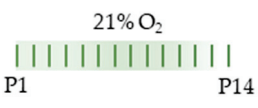
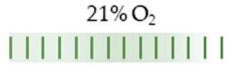
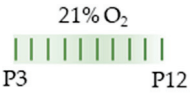
Species	Oxygen Exposure	Caffeine	Caffeine and the Immature Lung		Ref.
					
 C57Bl/6 mice		1 g/L lactate dam P0–P14	**Pulmonary damage** none	**Pulmonary side effects** reduced AdoRA2a expression	[101]
 C57Bl/6 mice		20 mg/kg i.p. P0–P4	**Pulmonary damage** none	**Pulmonary side effects** induced HIF mapped transcripts induced vascular-associated transcripts weight gain (male)	[102]
 SD rat		20 mg/kg i.p. P2–P10/P21	**Pulmonary damage** increased ER-phagy associated protein activated UPR (linked to fibrosis and inflammation) decreased blood vessel formation	**Pulmonary side effects** n/a	[103]
 C57Bl/6 mice		25 mg/kg i.p. P0–P13	**Pulmonary damage** decreased phosphorylation Smad2 (linked to epithelial remodeling)	**Pulmonary side effects** n/a	[104]
 SD rat		20 mg/kg i.p. P2–P21	**Pulmonary damage** n/a	**Side effects** weight loss	[105]
 SD rat		10 mg/kg p.o. P1–P14	**Pulmonary damage** n/a	**Pulmonary side effects** n/a	[124]

Table 8. *Cont.*

Species	Oxygen Exposure	Caffeine	Caffeine and the Immature Lung		Ref.
					
 FVB/n mice		20 mg/kg i.p. P1 10 mg/kg i.p. P2–P15	**Pulmonary damage** pro-apoptotic pro-inflammatory with MΦ infiltration and chemokine-induction reduced surfactant protein C transcript reduced AEC II	**Pulmonary side effects** n/a	[108]
 SD rat		15 mg/kg p.o. P3–P12	**Pulmonary damage** n/a	**Pulmonary side effects** increased minute ventilation increased breathing frequency	[122]

The color in the schematic experimental designs shows normoxia (21% O_2, green) with a horizontal line during sustained caffeine administration or in vertical lines to indicate the frequency of caffeine administration.

Since the groups without oxygen exposure are included in lung injury models and this corresponds to the developmental stage, caffeine application began on the day of birth (P0) in six studies. Caffeine administration was initiated in the study by Teng et al. [103] at P2 and, in the study by Julien et al. [122], at P3. The analysis was mainly performed after the last caffeine administration, except for in the studies by Teng et al. [103] and Dumpa et al. [102], who kept the pups without caffeine administration until the third week of life and the 12th week of life, respectively. The caffeine concentrations were in the range of from 10 mg/kg to 25 mg/kg for clinical use, except in the study by Dayanim et al. [108], which used a bolus administration of 20 mg/kg followed by 10 mg/kg, and the study by Chen et al. [101], in which the pups were supplied with caffeine via the lactating mother.

Caffeine exhibited no beneficial effects on the development of the lungs in any study, rather, the opposite effects were observed. Dayanim et al. [108] demonstrated, in newborn mice, that caffeine had a pro-apoptotic effect and also showed pro-inflammatory effects via increased infiltration with macrophages and induction of chemokine expression. Caffeine also decreased the level of AECII and reduced the transcription of surfactant protein C [108]. The translation of ER-phagy-associated proteins, which are involved in the continuous remodeling of the endoplasmic reticulum (ER) via selective autophagy [125], was induced by caffeine in the study by Teng et al. [103].

This was accompanied by an activation of the unfolded protein response (UPR), a reaction of the ER to oxidative stress [126], as well as reduced blood vessel formation. Reduced Smad2 phosphorylation was demonstrated in the study by Rath et al., which is associated with epithelial remodeling [127]. Side effects of caffeine treatment were also reduced AdoRA2a expression [101] and induction of HIF-mapped and vascular-associated transcripts [102]. The effect of caffeine on body weight was both increasing [102] and decreasing [105], whereby caffeine increased minute ventilation and respiratory rate as a side effect [122].

3.5.2. Caffeine in the Developing Brain without Oxidative Stress

To obtain an overview of the effects of postnatal administered caffeine on brain development, ten studies were evaluated (Table 9) [109,110,128–135]. Nine of these stud-

ies used rats (Wistar, Sprague Dawley, and Long Evans), and one used mice (species not reported).

Table 9. Caffeine effects in the developing brain under standard conditions.

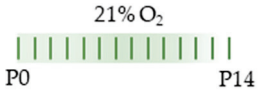
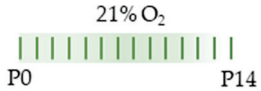
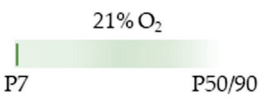
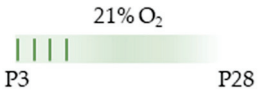
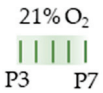
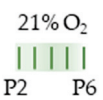
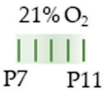
Species	Oxygen Exposure	Caffeine	Caffeine and the Developing Bain		Ref.
					
 SD rat		20 mg/kg i.p. P0 5 mg/kg i.p. P1–P14	**Neurodegeneration** pro-inflammatory	**Neuronal side effects** hypermyelination	[109]
 SD rat		LoC: 20 mg/kg i.p. P0 5 mg/kg i.p. P1–P14 HiC: 80 mg/kg i.p. P0 20 mg/kg i.p. P1–P14	**Neurodegeneration** LoC: pro-oxidant neuronal disorganization HiC: pro-oxidant pro-apoptotic pro-necrotic neuronal degeneration and disorganization	**Neuronal side effects** LoC: hypermyelination increased neurons per area and dendrites per neuron reduced AdoRA1 and AdoRA2a expression HiC: hypermyelination increased neurons per area and dendrites per neuron reduced AdoRA1 and AdoRA2a expression	[110]
 Wistar rat		10 mg/kg s.c. P7	**Neurodevelopment** hyperlocomotion reduced social interaction reduced contextual fear conditioning	**Neuronal side effects** n/a	[128]
 SD rat		50 mg/kg s.c. P3–P6	**Neurodegeneration** pro-apoptotic impaired mitochondrial dysfunction	**Neuronal side effects** n/a	[129]
 Wistar rat		10 mg/kg i.p. P6	**Neurodegeneration** reduced hippocampal proliferation capacity reduced differentiation potential in hippocampal neural linage	**Neuronal side effects** increased neurotrophin expression	[130]
 Long Evans rat		20 mg/kg s.c. P3 15 mg/kg s.c. P4–P7	**Neurodegeneration** reduced neurons (Hth and CA1) pro-apoptotic (CA1, Hth, Th, WM)	**Neuronal side effects** n/a	[131]
 SD rat		20 mg/kg i.p. P2 15 mg/kg i.p. P3–P6	**Neurodegeneration** none	**Neuronal side effects** induced early maturation adenosinergic system (Hth, brain stem) induced AdoRA1 and AdoRA2a expression	[133]

Table 9. *Cont.*

Species	Oxygen Exposure	Caffeine	Caffeine and the Developing Bain		Ref.
					
 mice		10 mg/kg i.p. P3 2.5 mg/kg i.p. P4–P10	**Neurodegeneration** reduced proliferation SVZ and WM reduced astrocytogenesis cortex and WM	**Neuronal side effects** n/a	[134]
 Wistar rat		10 mg/kg s.c. P7–P11 20 mg/kg s.c. P7–P11	**Neurodevelopment** reduced motoric performance hyperactivity	**Neuronal side effects** n/a	[135]

The color in the schematic experimental designs shows normoxia (21% O_2, green) with vertical lines to indicate the frequency of caffeine administration.

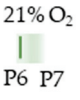
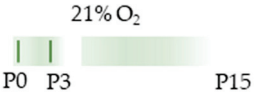
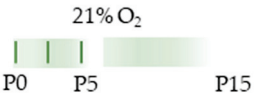
The caffeine application start points and duration were highly variable in these experiments. Two studies started at P0, with daily application until P14. Caffeine was dosed via a bolus (P0) of 20 mg/kg [109,110] or 80 mg/kg (i.p.) [110], followed by a constant dose of 5 mg/kg or 20 mg/kg. Kasala et al. [129] administered 50 mg/kg subcutaneously over four days, starting at P3, and analyzed the effects at P28. Two studies [131,133] had similar experimental designs, which administered caffeine at P2/P3 (20 mg/kg) and subsequently for five days (P6/P7, 15 mg/kg), whereby the routes of administration (s.c./i.p.) differed. The study by Desfrere et al. [134] also started at the same age (P3), here with mice, with a caffeine concentration of 10 mg/kg (i.p.), continuing until P10 (2.5 mg/kg (i.p.). Tchekalarova et al. [135] performed the experiments with two different caffeine doses (10 mg/kg and 20 mg/kg) at P7 with daily administration (s.c.) until P11. Caffeine was administered once to the rat pups in three studies at P6 or P7 [128,130,132], with a short observation period of 24 h (up to P7) or with additional examination times at P50/P90 [128].

Caffeine exhibited pro-oxidative [110], pro-inflammatory [109,132], pro-apoptotic [110,129,132], and/or pro-necrotic effects, resulting in neuronal disorganization, neurodegeneration [110], and mitochondrial dysfunction at the cellular level [129]. In detail, caffeine impaired neuronal proliferation [130,134], hippocampal neurogenesis [130], neuronal quantity [131], as well as astrocytogenesis [134]. In subsequent behavioral tests, the caffeine-treated animals showed hyperlocomotion, reduced social interaction, and altered anxiety behavior [128], as well as reduced motor performance and hyperactivity [135]. In addition to hypermyelination [109,110] and neurotrophin induction [130] after caffeine treatment, a modulation of AdoRA1 and AdoRA2a gene expression was also demonstrated, either with activation [132,133] or inhibition [110], accompanied by an early maturation of the adenosinergic system [133].

3.5.3. Caffeine in the Lung–Brain Axis without Oxidative Stress

Based on the studies already presented by our group (see Table 10), these also allow us to observe the caffeine-treated rat pups under normoxic environmental conditions without the presence of oxidative stress as a noxious agent [38,39,68–71,75]. All studies used accompanying caffeine-treated animals, which were analyzed in parallel.

Table 10. Caffeine effects in the immature lung and the developing brain under standard conditions.

Species	Oxygen Exposure	Caffeine	Caffeine and the Immature Lung and the Developing Brain		Ref.
					
 Wistar rat	 21% O_2 P6 P7	10 mg/kg i.p. P6	**Pulmonary damage** pro-inflammatory	**Neurodegeneration** pro-inflammatory modulated NfκB signaling pathway reduced hippocampal proliferation reduced hippocampal neurogenesis inhibited physiological apoptosis	[38,69,75] The studies use the identical cohorts.
	 21% O_2 P6 P8	10 mg/kg i.p. P6			
 Wistar rat	 21% O_2 P0 P3 P15	10 mg/kg i.p. P0–P2	**Pulmonary damage** pro-inflammatory **Pulmonary side effects** modulated NfκB/Nrf2 signaling pathway and AdoRa1 expression **Side effects** gain loss	**Neurodegeneration** reduced hippocampal neurogenesis reduced hippocampal proliferation reduced neurotrophin expression **Neuronal side effects** increased AdoRA1, AdoRA2a, and AdoRA2b expression modulated cerebellar neurogenesis and migration induced cerebellar proliferation	[39,68,70,71] The studies use the identical cohorts.
	 21% O_2 P0 P5 P15	10 mg/kg i.p. P0–P4			

The color in the schematic experimental designs shows normoxia (21% O_2, green) with vertical lines to indicate the frequency of caffeine administration.

We focused the analyses on a single application (P6) of caffeine with short-term survival (P7/8) [38,69,75] and a multiple application from the day of birth (P0) to P3 or P5 [39,68,70,71], to then analyze survival without further caffeine administration up to P15 in addition to these study time points. Caffeine at 10 mg/kg (i.p.) was administered.

Both a single dose of caffeine and multiple two-day near-birth administrations mediated pro-inflammation in the postnatal lungs of rat pups [68,75], with demonstrable modulation of AdoRA1 gene expression [68]. Prolonged administration of caffeine reduced body weight for as long as caffeine was administered but normalized by the second week of life without further caffeine administration [68].

Caffeine also impaired neuronal development and induced pro-inflammatory effects. A single dose of caffeine induced proinflammatory cytokines and modulated the NfκB signaling pathway [38]. Similarly, this single administration of caffeine was sufficient to impair hippocampal neurogenesis and proliferation and prevent physiological apoptosis [69]. Similar effects can be demonstrated for repeated doses starting at P0. Here, too, hippocampal neurogenesis and proliferation capacity are reduced [71]. Giszas et al. [70] showed that neurogenesis and migration are modulated in the cerebellum, and, in addition to induction of AdoRA1, AdoRA2a, and AdoRA2b gene expression, cerebellar proliferation was upregulated.

4. Discussion

4.1. Caffeine, the 'Gamechanger' for Oxidative Stress Injury in the Brain–Lung Axis

Caffeine demonstrated very impressive results in the sum of all these presented studies indicating that, under oxidative stress in any model, the vulnerable, developing brain and the postnatal still immature lung in the rodent model can benefit to a high degree from the radial scavenger caffeine, and this can protect against the effects of ROS. Cerebral and cerebellar perfusion is developmentally more susceptible to reperfusion injury and oxidative stress [136]. Oxidative stress can have multiple effects on the immature organism of a premature infant [62,137]. There are molecular mechanisms that demonstrate a link between oxidative stress and the effects of caffeine. Clinical and experimental studies show this primarily through protective effects on neurological outcomes and improved lung protection [38,39,68–71,75,138–143].

The proven effects of oxygen toxicity and the effect of caffeine, combined with the damage models, are displayed schematically in Figure 1. In detail, ROS caused increased apoptosis rates, inhibited proliferation capacities, and impaired the impairment of differentiation stages in relevant progenitor cells through the oxidation of macromolecules [38,39,110]. Lipid peroxidation and peroxidative membrane lipid damage triggered by ROS or corresponding initiators lead to critical cell damage and thus cell death. In addition, this process causes inflammation [144]. DNA lesions triggered by oxidative stress were mediated via various signaling pathways and induced inflammation and apoptosis [145]. If uninterrupted, this can lead to irreversible damage and the pathogenesis of diseases of prematurity by impairing structure and function [56,138,140,146,147].

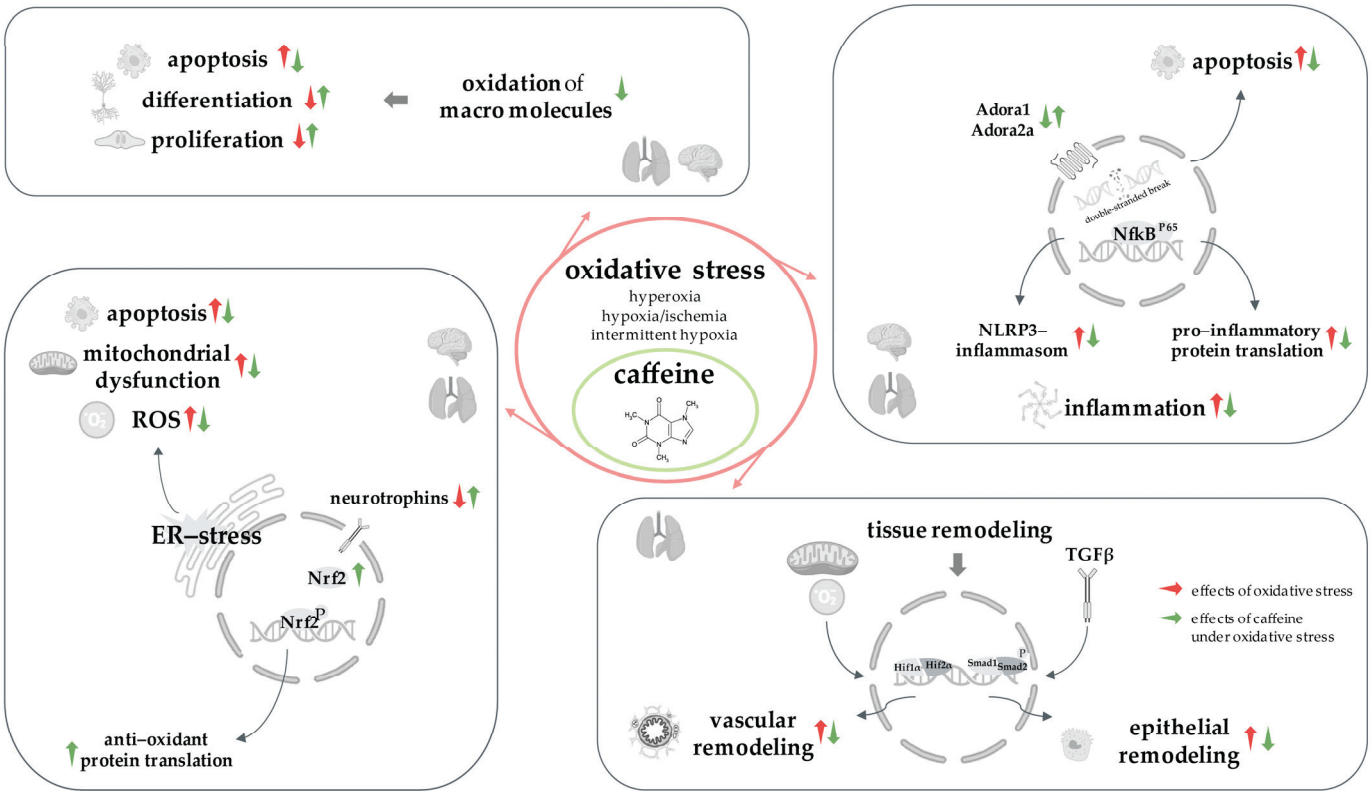


Figure 1. Oxidative stress and caffeine signaling in the brain–lung axis. Oxidative stress-mediated cellular signaling pathways in the immature lung and developing brain, with caffeine as an adequate counteractant to oxygen toxicity.

Teng et al. [103] were able to identify ER stress as a co-triggering factor for oxygen damage in a hyperoxia model of the newborn rat. Disturbances of physiological ER functions caused an accumulation of newly synthesized unfolded and misfolded proteins

(UPR) [126]. The combination and responsiveness of different UPR signaling pathways after ER stress determines cell survival or death [148,149]. If the ER stress-inducing factors cannot be rebalanced, the UPRs trigger pro-apoptotic signaling pathways. ER functions, such as the maintenance of mitochondrial function and the appropriate limitation of oxidative stress, are closely related to alveologenesis [150]. ER and oxidative stress are two components that have been observed in BPD animal models [151,152]. Induction of ER stress with oxidative stress plays a non-negligible mechanistic component in injury to the developing brain [153].

Nrf2 and NfκB are important factors in an adequate response to oxidative stress and inflammation. They act independently of each other on intrinsic and extrinsic agents, but they can certainly interact [154]. Both transcription factors in oxidative stress models are influenced by the induction or inhibition of the translation of anti-oxidative, but also pro-inflammatory response molecules, and the formation of NLRP3 inflammasomes [38,39,101]. The essential function of NfκB has been described in many experimental studies, whereby the neonatal lung appears to express higher levels of protection against oxidative damage [155] and more protected alveologenesis upon sustained activation of this transcription factor [156]. The redox-sensitive transcription factor Nrf2 mediated protection against oxidative stress through transcriptional activation of various antioxidant enzymes via the antioxidant response element (ARE) [157,158].

In a clinical study, preterm infants had lower BDNF levels than term-born infants [159], with lower BDNF often correlating with reduced neurodevelopmental outcomes [160,161]. BDNF, as well as other neurotrophic factors, plays a significant role in the normal development and function of both the respiratory and nervous systems [162–164]. Oxidative stress and inflammation are important factors, but they can influence neurotrophins. Damage models of the immature lung and brain also demonstrate this through downregulated neurotrophic factors [70,71,165].

Epithelial damage and fibrosis are consequences of BPD [166]. Alveolar growth is controlled by angiogenesis and vascular branching [167]. Various growth factors play an essential role in this process, such as TGFβ. Dysregulated TGFβ causes a simplification of lung morphology under hyperoxia as well as hypoxia [168,169]. Vascular mediators, such as VEGF, mediate the growth and remodeling of endothelial cells in the lung [170]. The impairing effect of oxidative stress on epithelial and vascular remodeling, as well as the impairment of pulmonary alveolar formation by a simplified structure of the lung, were displayed in numerous experimental studies cited here [39,101–107].

Caffeine, with its pleiotropic effect, was able to effectively counteract these effects triggered by oxidative stress via different oxidative stress-associated models. As can be seen in the overviews of the individual oxygen damage models (Tables 1–7), the neuronal and pulmonary protective properties of caffeine are independent of oxygen partial pressure, duration, and generally also of the application. In connection with the proven antioxidant effects via classical biomarkers, this, again, strongly supports this property. However, it is also evident that caffeine modulates the adenosine receptors under oxidative stress so that the caffeine effect is not a direct effect via a single signaling cascade. The response of caffeine to oxygen toxicity is mediated via anti-oxidative, anti-inflammatory, and anti-apoptotic signaling cascades in the brain–lung axis [24,137].

The pulmonary effects are associated with improved microvascularization and alveologenesis [39,101–103,105–107]. Caffeine also influenced ER stress and reduced mitochondrial dysfunction, and it mediated this via fibrosis- and vascular-associated transcripts [103]. In fact, these studies showed no effect of caffeine on oxidative stress [104,108]. Caffeine, as a non-selective adenosine antagonist for adenosinergic receptors, also modulated the expression of the receptors in lung tissue, so that this influence could not be neglected [101,171,172]. Clinically, the spectrum of effects of caffeine on the pulmonary outcome of preterm infants has been demonstrated very well and reflects the experimental findings, as well as the improved respiratory function and reduction in AOP frequency [122].

Schmidt et al. [4] were initially able to demonstrate an improved neuronal outcome, primarily through a reduction in the risk of cerebral palsy, as a result of caffeine in the CAP study. Many clinical studies followed (reported in the introduction) and were supplemented by experimental studies (reported in the results section), thus increasing the robustness of evidence regarding the neuroprotective effect of caffeine. The anti-oxidative, anti-inflammatory, and cell death-reducing effects of caffeine also prevailed here [38,69,109,110,114,115,117,120] and could be displayed more concretely for the protection against oxidative stress for essential processes such as neurogenesis, dendritogenesis, and myelination [38,69–71,109,110,112,114,115,117,153]. Improved EEG activity and improved motor function and cognition [116,118,119,121] also suggested a protective effect of caffeine against functional deficits. The influence of caffeine on the antioxidant response, as well as the modulation of adenosine receptors in the brain, indicated similar mechanisms of action for caffeine. Systemically administered caffeine in a single dose or over several days in the vulnerable phase of lung and brain development can reconcile this very well [38,39,68–71,75].

Is caffeine a 'game-changer'? In the overarching view, caffeine is the 'game-changer' in oxidative stress-relevant damage models and in the clinical setting of premature infants who are exposed to a hyperoxic environment due to premature translation, but also triggered by other unavoidable stressors that cause or intensify oxidative stress [173].

4.2. Caffeine, Forever and Ever?

Caffeine is and was used to reduce the frequency of AOP and is the standard drug in the NICU. Side effects such as lower mortality, less BPD, less cerebral palsy, and improved respiratory and motor–cognitive outcomes are well recognized [174]. The use of caffeine therapy is quite variable in the clinic and requires its use in only ventilated premature infants [175], only in premature infants with AOP [176], and, as intensively discussed, with onset within the first three days of life; each case requires different dosing [177]. This variability can certainly also be explained by the increased respiratory instabilities, such as intermittent hypoxia and increased AOP frequency, which correlate with lower gestational age [178]. Currently, caffeine therapy is continued until about 34 corrected weeks of pregnancy (postmenstrual age, PMA) [4,179], while birth before 27 weeks of gestation, BPD, or NEC prolongs the achievement of adequate physiological development [180,181].

Clinical studies on prolonged administration of caffeine are rather scarce. Rhein et al. [182] found that prolonged caffeine therapy for up to 36 weeks of PMA reduced intermittent hypoxia episodes. A continuation of these studies at constant caffeine levels in the therapeutic range demonstrated a reduction in persistent intermittent hypoxia up to 38 weeks PMA [183]. This clinical trial by Rhein et al. was also included in a recent Cochrane analysis [184]. In addition to caffeine therapy in relation to postmenstrual age, two studies were also included that investigated the non-occurrence of symptoms in relation to postmenstrual age. Here, too, early discontinuation of caffeine may be associated with a possible increase in IH episodes [185,186].

Caffeine is a free radical scavenger [37,187], but caffeine also antagonizes cellular adenosine receptors, which are ubiquitously expressed in the brain and respiratory tract [188]. Thus, caffeine also has the potential to trigger effects that could lead to side effects [18–22], such as the induction of a proinflammatory cytokine profile [21] or reduced cerebral oxygenation [19]. When caffeine was administered postnatal to rodents that were in a comparable phase of development to premature infants in the last trimester of pregnancy, adverse effects occurred. In studies of the immature lungs and brains of identical animals, an important finding was induced pro-inflammation [38,68,75], which was often associated with increased cell death rates and induction of the NfκB signaling pathway. Dayanim et al. [108] observed pulmonary infiltration with macrophages and chemokines. Pro-inflammatory responses have also been demonstrated in studies of the developing brain [109,130,132]. These were mostly transient effects, which, in turn, triggered increased oxidative stress [110]. Nevertheless, developmental disabilities in extremely premature newborns are most likely multifactorial but are definitely associated with inflammation at

the time of birth. The causal assumption is that transient systemic inflammation can also lead to neurological deficits [189–191].

Caffeine, as a single 'second hit', has been demonstrated to lead to structural and functional impairments (Tables 8–10), such as neuronal disorganization and degeneration [110], reduced vascularization [103], and impaired proliferation and differentiation potential of neuronal progenitor cells [69–71,132,134]. Long-term observations then mediated altered behavior and hyperactivity after caffeine treatment [128,135], as well as tachypnea [122]. As expected, caffeine also modulated the expression of adenosine receptors [68,101,110,132]. The involvement of caffeine in the inflammatory response via the adenosine receptors has been demonstrated and also revealed a correlation between caffeine levels and cytokine levels [21,192]. Similarly, caffeine plasma levels in preterm infants are variable but correlate well with the PMA. Due to the immaturity of the hepatic system of preterm infants, caffeine remains almost unmetabolized while renal development continues [193]. In combination with the different expressions as well as the affinity of the adenosine receptors [194] for caffeine, the physiological effects appear to be divergent. Due to the lack of long-term studies in both experimental and clinical settings, it is not possible to provide a comprehensive assessment at this time.

5. Conclusions

Caffeine is one of the most commonly used drugs in neonatal intensive care. The benefits of caffeine are evident in terms of reducing apnea of prematurity and minimizing the risk of death, as well as neurological and respiratory comorbidities. In vivo models and premature infant cohorts have demonstrated these effects. These animal studies evaluated caffeine effects in various preterm infant-associated models, including drugs, inflammation, and oxidative stress. Second hits are unavoidable in preterm infants, and the identification of therapeutic strategies to minimize the resulting adverse effects on the immature organism is essential for translation into the clinical setting.

Caffeine therapy is currently administered up to around 34 corrected gestation weeks. The arguments for this are quite sound, as the developmental milestones, such as the maturation of the respiratory system and thus the decrease in the frequency of central apnea, as well as the increase in antioxidant capacity, have been reached. The consideration of prolonging caffeine therapy seems reasonable to increase the response to ongoing oxidative challenges, such as intermittent hypoxia or oxidative stress-inducing triggers, of the preterm infant. Robust clinical preterm and neonatal studies are currently scarce and cannot yet support treatment decisions. However, experimental studies in rodents have shown that caffeine per se as a single 'second hit' does not appear to be entirely harmless. In combination with established damage models for oxidative stress, caffeine is a 'game-changer' and the caffeine effects can confirm the clinical observations. Of course, the translation into human application must be discussed. The significance seems limited, as, despite comparable ontogenetic phases of neuronal and pulmonary development from rodents to humans, rodents have an already mature antioxidant enzyme system. Nevertheless, it is reasonable to assume that, if the beneficial effects of caffeine under oxygen toxicity are comparable in in vivo models of therapeutic effects in preterm infants, the adverse effects without induced oxidative stress, such as the induction of pro-inflammatory cytokine levels, are also likely to occur. Transient systemic inflammation is a possible predictor of deficits in long-term outcomes. Caffeine therapy of the preterm infant beyond the currently recommended 34 corrected gestation weeks does not appear to be indicated based on current data and requires a high level of further clinical and experimental studies to further ensure the safety of caffeine therapy.

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antioxidants

Review

Melatonin Use during Pregnancy and Lactation Complicated by Oxidative Stress: Focus on Offspring's Cardiovascular–Kidney–Metabolic Health in Animal Models

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Abstract: Cardiovascular–kidney–metabolic (CKM) syndrome has emerged as a major global public health concern, posing a substantial threat to human health. Early-life exposure to oxidative stress may heighten vulnerability to the developmental programming of adult diseases, encompassing various aspects of CKM syndrome. Conversely, the initiation of adverse programming processes can potentially be thwarted through early-life antioxidant interventions. Melatonin, originally recognized for its antioxidant properties, is an endogenous hormone with diverse biological functions. While melatonin has demonstrated benefits in addressing disorders linked to oxidative stress, there has been comparatively less focus on investigating its reprogramming effects on CKM syndrome. This review consolidates the current knowledge on the role of oxidative stress during pregnancy and lactation in inducing CKM traits in offspring, emphasizing the underlying mechanisms. The multifaceted role of melatonin in regulating oxidative stress, mediating fetal programming, and preventing adverse outcomes in offspring positions it as a promising reprogramming strategy. Currently, there is a lack of sufficient information in humans, and the available evidence primarily originates from animal studies. This opens up new avenues for novel preventive intervention in CKM syndrome.

Keywords: oxidative stress; hypertension; metabolic syndrome; developmental origins of health and disease (DOHaD); kidney disease; nitric oxide; melatonin; antioxidants

1. Introduction

Increasing recognition of the pathological interconnections among metabolic risk factors like obesity and diabetes, chronic kidney disease (CKD), and cardiovascular disease (CVD) has given rise to the conceptualization of cardiovascular–kidney–metabolic (CKM) syndrome [1]. For the first time, the 2023 Scientific Statement from the American Heart Association defines CKM syndrome as a systemic disorder marked by pathophysiological interactions among metabolic risk factors, CKD, and the cardiovascular system. This interaction results in multiorgan dysfunction and a heightened risk of adverse cardiovascular and renal outcomes [1]. CKM syndrome is classified into four distinct stages, ranging from stage 0 to stage 4. These stages likely represent varying degrees of progression and severity within the complex spectrum of this disease. Various key components manifest at different stages, contributing to the varied degrees of progression and severity within the complex spectrum of CKM syndrome.

While the global disease burden of individual metabolic diseases, CKD, and CVD is widely recognized [2–5], the complex and mutually reinforcing detrimental relationships among them remain largely unclear. Epidemiological studies suggest a multidirectional

connection among these conditions. For instance, individuals with CKD face an elevated risk of developing CVD [6]. Comorbidities associated with CKD, such as obesity, diabetes, and hypertension, constitute significant components of metabolic syndrome [7]. Additionally, cardiorenal syndrome is a recognized condition in which dysfunction of either the heart or kidneys can impair function in the other organs [8]. Furthermore, metabolic syndrome, a cluster of conditions, collectively amplifies the susceptibility to CVD [9]. Recently, the concept of metabolic syndrome has evolved into one of cardiometabolic syndrome, encompassing characteristics of metabolic syndrome along with traditional CVD factors [10]. Consequently, it is plausible that CVD, CKD, and metabolic diseases may interact at the pathophysiological level, leading to clinical overlap between these health conditions [11].

Approximately 40% of adults in the United States are estimated to be affected by CKM syndrome [12]. Given that CKM syndrome impacts nearly all organ systems, there is a substantial global burden of compromised cardiovascular–kidney–metabolic health. Although managing the entire syndrome holistically, rather than focusing on individual diseases, is recommended for optimal care [12], a comprehensive therapeutic guideline is still pending. It is noteworthy that placing emphasis on early prevention has the potential to alleviate the burden associated with CKM syndrome. Recognizing the interplay between CKM diseases is crucial for adopting a more holistic approach to CKM care, moving beyond the isolated treatment of individual conditions. This broader perspective holds promise for enhancing global health outcomes in the future.

An expanding body of literature indicates that unfavorable environmental conditions during pregnancy and early infancy may increase the susceptibility to adult diseases [13], encompassing all facets of CKM syndrome. Termed the Developmental Origins of Health and Disease (DOHaD), this theory posits that a developing fetus responds to environmental challenges by making structural and functional adaptations, thereby increasing the risk of developing chronic diseases later in life [14]. Although our understanding of the pathophysiological mechanisms of DOHaD remains incomplete, oxidative stress plays a role in the etiology of many DOHaD-related diseases [15].

Oxidative stress arises from an imbalance between the production of harmful reactive oxygen/nitrogen species (ROS/RNS) and the antioxidant system's ability to detoxify them [16]. Both human and experimental studies provide evidence supporting the involvement of oxidative stress in various aspects of CKM syndrome [17–19] (Figure 1). Conversely, the administration of antioxidants has been reported to ameliorate CKM syndrome [20–22]. Early-life interventions, referred to as reprogramming, have the potential to reverse detrimental programming processes and alleviate the onset of adult diseases [23]. The use of antioxidants during pregnancy and lactation has demonstrated benefits as a reprogramming strategy for preventing numerous adult diseases [24].

Melatonin, primarily secreted by the pineal gland during nighttime, serves as a versatile hormone [25]. Alongside its metabolites, melatonin exhibits antioxidant properties, positioning this molecule as an intrinsic safeguard against various disorders associated with oxidative stress [26,27]. In recent times, melatonin has found applications in the treatment of pregnant women and various pediatric conditions [28–30]. In this context, we will delve into the existing knowledge regarding the links among melatonin, maternal oxidative stress, and the development of offspring CKM syndrome.

A comprehensive literature review was conducted by querying the databases Embase, MEDLINE, and the Cochrane Library to identify studies published in English. The search utilized relevant keywords pertaining to melatonin, oxidative stress, DOHaD, and CKM syndrome. The employed search terms included "metabolic syndrome", "hypertension", "dyslipidemia", "hyperlipidemia", "obesity", "diabetes", "insulin resistance", "hyperglycemia", "liver steatosis", "chronic kidney disease", "cardiovascular disease", "atherosclerosis", "heart failure", "cardiorenal syndrome", "developmental programming", "DOHaD", " free radicals", "offspring", "progeny", "mother", "prenatal", "nitric oxide", "oxidative stress", "pregnancy", "reprogramming", "reactive oxygen species", "reactive nitrogen species", and "melatonin". Additionally, we scrutinized the reference lists of

articles to identify any additional references relevant to this review. The search concluded on 30 November 2023.

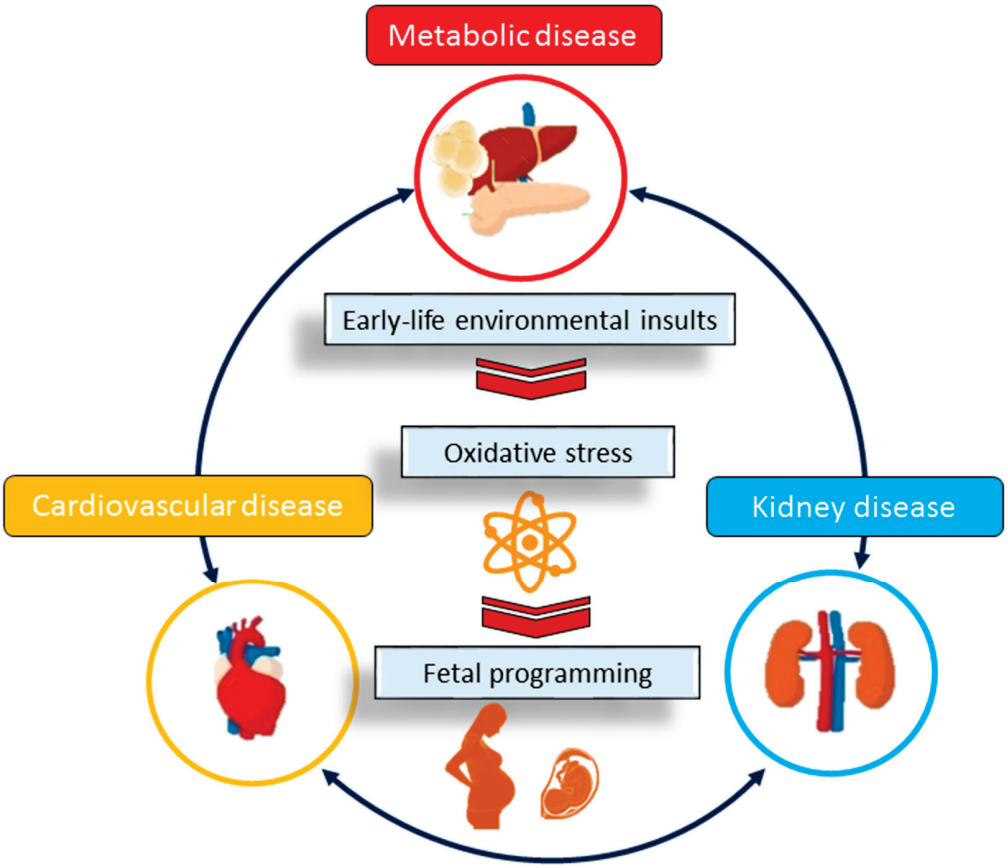


Figure 1. Schematic representation of the interrelationships between early-life oxidative stress, fetal programming, and cardiovascular–kidney–metabolic syndrome in later life.

2. Oxidative Stress

2.1. ROS, RNS, NO, and Antioxidants

Oxidative stress results from an imbalance between prooxidants and antioxidants [16]. The detrimental effects of oxygen/nitrogen free radicals and non-radical reactive species, collectively referred to as ROS/RNS, can lead to potential biological oxidative damage [31]. Cellular nitric oxide (NO) can interact with ROS, giving rise to various RNS implicated in nitrosative damage. Among the crucial reactive species are the superoxide anion radical (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). Major enzymatic sources of ROS include the electron transport chain complexes in mitochondria, NADPH oxidase (NOX), and xanthine oxidase (XO). Additionally, NO can be produced through three isoforms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [32]. Asymmetric and symmetric dimethylarginine (ADMA and SDMA, respectively), inhibitors of NOS, have the potential to uncouple NOSs, leading to peroxynitrite generation. This not only diminishes NO production, but also intensifies oxidative stress [33].

Conversely, excessive ROS/RNS can be counteracted by antioxidant systems, encompassing enzymatic components such as superoxide dismutase (SOD) and non-enzymatic antioxidants like glutathione [34]. Throughout pregnancy, a delicate balance is established among ROS, RNS, and antioxidants, crucial for determining normal fetal growth and development.

2.2. Oxidative Stress during Pregnancy

Throughout pregnancy, the physiological generation of ROS plays a crucial role in various developmental processes, encompassing oocyte maturation [35], embryo implantation [36], placental differentiation [37], and fetal development [38]. The fetal oxygen requirement varies across different trimesters, with low levels during the initial trimester and a subsequent increase in response to rapid fetal weight gain and the formation of fetal–placental circulation [39].

Compromised pregnancies, marked by maternal illnesses and adverse conditions, are associated with oxidative stress. These conditions include preeclampsia, maternal smoking, obesity, diabetes, placental insufficiency, preterm labor, and intrauterine growth retardation (IUGR) [40]. For instance, current evidence suggests that the pathogenesis of preeclampsia involves increased lipid peroxidation products, elevated nitrotyrosine immunostaining, and reduced antioxidant enzyme activities [41]. Another significant indicator is ADMA. There is growing support for the association of high ADMA levels with conditions such as preeclampsia [42], gestational diabetes [43], IUGR, and placental insufficiency [44]. Furthermore, studies have reported elevated levels of ROS or oxidative stress by-products and decreased antioxidant levels in cases of preterm labor [45].

2.3. Animal Models of Oxidative-Stress-Associated Offspring CKM Syndrome

Accumulating evidence underscores the pathogenic interplay between oxidative stress and various facets of CKM syndrome, including obesity [46], kidney disease [47], CVD [48], diabetes [49], nonalcoholic fatty liver disease (NAFLD) [50], and hypertension [51]. However, there is a lack of information regarding oxidative stress during human pregnancy that may be associated with offspring CKM syndrome in childhood or adulthood.

Several recent animal studies emphasize the significance of early-life oxidative stress concerning specific components of CKM syndrome, such as kidney disease or hypertension, and these have been comprehensively reviewed elsewhere [24,52]. However, a notable gap exists in studies directly addressing the impact of early-life oxidative stress on all CKM traits in adult offspring. Therefore, Table 1 predominantly compiles findings from animal models exhibiting at least two components of CKM syndrome in offspring that are associated with oxidative stress [53–100]. Moreover, this review focuses on rat models to facilitate relevant comparisons of major CKM syndrome components throughout the lifespan.

Table 1. Animal models displaying CKM syndrome in adult offspring related to oxidative stress.

Model	Timing	Age at Evaluation (Weeks)	Features of CKM Syndrome	Oxidative Stress	References
Caloric restriction, 50%	Throughout gestation and lactation	12–16	Hypertension, insulin resistance, and kidney disease	↑Renal 8-OHdG expression, ↑3-NT, ↑ADMA, ↓NO	[53–55]
Protein restriction, 6–9%	Gestation	12	Hypertension, insulin resistance, and kidney disease	↑F_2-isoprostane, ↑3-NT, ↓SOD and GPX activity, ↓glutathione	[56–58]
Maternal high-fructose diet, 60%	Throughout gestation and lactation	12–52	Hypertension, obesity, insulin resistance, and dyslipidemia	↑Renal 8-OHdG expression ↑MDA, ↑Brain NADPH-oxidase expression; ↑ROS, ↓NO	[59–63]
Maternal high-fat diet, 58%	Throughout gestation and lactation	16	Hypertension, obesity, insulin resistance, dyslipidemia, and kidney disease	↓SOD activity, ↑Renal MDA; ↑Renal 8-OHdG expression	[64–68]

Table 1. *Cont.*

Model	Timing	Age at Evaluation (Weeks)	Features of CKM Syndrome	Oxidative Stress	References
Maternal diabetes	Neonatal streptozotocin injection	12–16	Hypertension, obesity, insulin resistance, dyslipidemia, and kidney disease	↑Renal 3-NT and TBARS; ↑ROS, ↓SOD activity, ↓NO; ↑ADMA	[69–71]
Uteroplacental insufficiency	Bilateral uterine artery ligation on gestation	22–30	Hypertension, dyslipidemia insulin resistance, and kidney disease	↑Renal NADPH-oxidase dependent superoxide, ↑Urinary F_2-isoprostane level	[72–75]
Maternal chronodisruption	Gestation	12–52	Hypertension and insulin resistance	↑Brain ROS	[76–78]
Maternal stress	Perinatal dexamethasone administration	16–24	Hypertension, obesity, insulin resistance, and kidney disease	↓Gpx1 expression, ↑NADPH-oxidase, ↓Renal NO, ↑ADMA, ↑Renal 8-OHdG expression	[79–82]
Maternal chronic kidney disease	Throughout gestation and lactation	12	Hypertension and kidney disease	↑Renal 8-OHdG expression, ↓NO	[83,84]
Nicotine exposure	Perinatal nicotine exposure	20–32	Hypertension, hyperlipidemia, steatosis, and kidney disease	↑MDA, ↑3-NT, ↑NADPH oxidase, ↑4-NHE, ↓GPx1 activity	[85–88]
Ethanol exposure	Gestation	24	Hypertension and insulin resistance	↓SOD1, ↓CAT, ↓GPX, ↑NOX2	[89–91]
TCDD exposure	Gestation and lactation	12	Hypertension, cardiac hypertrophy, and kidney disease	↑Renal 8-OHdG expression	[92,93]
BPA exposure	Gestation and lactation	16–24	Hypertension, insulin resistance, and steatosis	↓SOD, ↓CAT, ↑Renal 8-OHdG expression, ↑ADMA, ↓NO	[94–96]
DEHP exposure	Gestation and lactation	12–21	Hypertension and insulin resistance	↑Renal ROS, ↑Renal 8-OHdG expression	[97–100]

TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin; BPA = bisphenol A; DEHP = di-n-butyl phthalate; ADMA = asymmetric dimethylarginine; 8-OHdG = 8-hydroxy-2′-deoxyguanosine; NO = nitric oxide; ROS = reactive oxygen species; 3-NT = 3-nitrotyrosine; TBARS = thiobarbituric acid; MDA = malondialdehyde; 4-NHE = 4-hydroxynonenal; CAT = catalase; GPX = glutathione peroxidase; SOD = superoxidase dismutase; NOX2 = NADPH oxidase 2.

Considering that one rat month is roughly equivalent to three human years [101], Table 1 provides the age of offspring outcomes, ranging from 12 to 52 weeks, in rats. This timeline corresponds to humans from young adulthood to middle age.

Imbalances in maternal nutrition play a significant role in inducing oxidative stress-related CKM syndrome in adult offspring. Various nutritional insults contribute to this, including calorie restriction [53–55], protein restriction [56–58], maternal high-fructose diet [59–63], and maternal high-fat diet [64–68]. Additionally, maternal illnesses and pregnancy complications can disrupt the oxidative balance, leading to the manifestation of CKM syndrome in offspring. For instance, maternal diabetes has been identified as a contributor to oxidative stress, linked to obesity, insulin resistance, hypertension, dyslipidemia, and kidney disease in adult offspring [69–71].

Several early-life risks have been associated with the development of CKM syndrome in offspring, including uteroplacental insufficiency [72–75], maternal chronodis-

ruption [76–78], maternal stress [79–82], maternal chronic kidney disease (CKD) [83,84], nicotine exposure [85–88], and ethanol exposure [89–91]. Furthermore, offspring CKM syndrome can be programmed by dams exposed to environmental chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [92,93], bisphenol A (BPA) [94–96], and di-n-butyl phthalate (DEHP) [99–101].

2.4. Mechanisms Underlying Oxidative Stress in CKM Syndrome

In developmental-origin-related CKM syndrome, a comprehensive array of oxidative stress mechanisms has been observed, encompassing an increased expression of ROS-generating enzymes, heightened ROS production, diminished antioxidant bioavailability, elevated peroxynitrite formation, an impaired ADMA–NO pathway, and augmented oxidative damage.

The elevated expression of ROS-generating enzymes and increased ROS production are linked to various CKM syndrome models, including those induced by a maternal high-fructose diet [62], uteroplacental insufficiency [75], maternal stress [82], nicotine exposure [85], and ethanol exposure [90]. Several CKM syndrome models also demonstrate reduced antioxidant bioavailability, such as decreased levels of glutathione [57], and compromised antioxidant enzyme activities [80], including SOD, glutathione peroxidase 1, and catalase.

The formation of 3-nitrotyrosine (3-NT), indicative of peroxynitrite-mediated protein modification [102], is correlated with CKM syndrome in offspring programmed by maternal caloric restriction [54], maternal low-protein diet [58], and prenatal nicotine exposure [87]. Additionally, various biomarkers reflecting oxidative damage to proteins, lipids, and DNA have been assessed in CKM syndrome animal models. These include malondialdehyde (MDA) [62], thiobarbituric-acid-reactive substances (TBARS) [70], F2-isoprostanes [75], 4-hydroxynonenal (4-NHE) [87], and 8-hydroxydeoxyguanosine (8-OHdG) [83,92].

Another contributing mechanism of oxidative stress in the pathogenesis of programmed CKM syndrome is the impaired ADMA–NO pathway. High levels of ADMA, inhibiting NO production and thereby impairing endothelial function, have been associated with the development of various CKM syndrome components. This is evident in models of caloric restriction [53], maternal diabetes [69], maternal stress [79], and perinatal bisphenol A (BPA) exposure [94].

2.5. Perinatal Use of Antioxidants as a Reprogramming Strategy

Given the pivotal role of early-life oxidative stress in influencing adverse outcomes in offspring, there has been a notable interest in exploring the perinatal use of antioxidants as a potential preventive strategy for adult diseases [15,24,52,103]. However, conclusive evidence of confirmed benefits from antioxidant supplementation, particularly in pregnant or lactating women, remains elusive in the majority of human studies [104]. The conflicting findings and challenges in interpreting clinical evidence may stem from factors such as the study population, the type of antioxidant, supplement timing and dosage, and the specific disease status deemed suitable for treatment. Hence, it is crucial to conduct additional research aimed at pinpointing the precise developmental stage (such as gestation or lactation) and unraveling organ-specific redox-sensitive signaling pathways associated with various maternal insults that contribute to CKM programming in animal models. This groundwork is essential before translating the findings into clinical applications.

Antioxidants can be classified as natural or synthetic [105]. Examples of natural antioxidants include vitamins A, C, and E, glutathione, polyphenols, carotenoids, and melatonin [106]. Although many natural antioxidants are widely used in humans [104], their benefits in pregnant and lactating women remain inconclusive.

Melatonin, a multifunctional hormone, is implicated in pregnancy and fetal development [107]. Research on melatonin has highlighted its crucial role in antioxidant defense against oxidative damage [26]. Notably, the utilization of melatonin during the perinatal period has been suggested as a reprogramming strategy to mitigate the risk of various

adult diseases associated with DOHaD [108]. This summary outlines the current evidence supporting the perinatal use of melatonin to protect against offspring CKM syndrome (Figure 2).

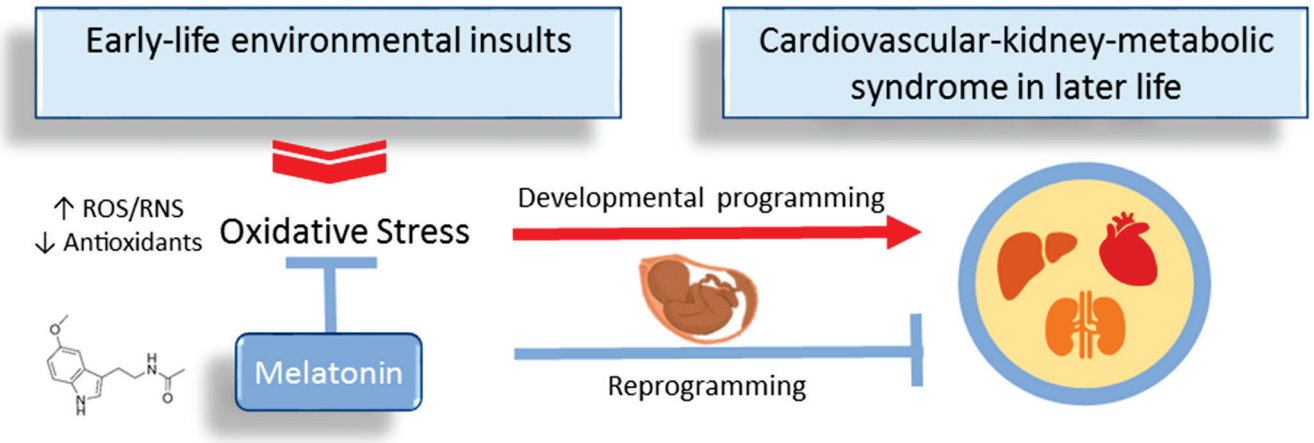


Figure 2. Overview of perinatal use of melatonin as a reprogramming strategy to prevent developmental programming of cardiovascular–kidney–metabolic syndrome.

3. Melatonin

3.1. Effects of Melatonin

Melatonin, initially identified in the bovine pineal gland [109], is predominantly synthesized by pinealocytes using tryptophan as a precursor [110]. While the pineal gland is a primary source, melatonin is also produced in various organs such as the retina, skin, gastrointestinal tract, and bone marrow [25]. The metabolism of melatonin occurs primarily in the liver and kidneys, with its primary urinary metabolite being 6-sulfatoxymelatonin [111].

Melatonin exerts its actions through binding to melatonin receptor-1 (MT1) and -2 (MT2), both of which are G-protein-coupled receptors widely distributed throughout the body [112]. Although there is some controversy regarding retinoid acid receptor (ROR) as a potential nuclear receptor for melatonin [113], the evidence remains inconclusive [114]. Melatonin also exhibits receptor-independent effects, such as activating cytoprotective pathways and functioning as a broad-spectrum antioxidant, possibly through its metabolites [111].

The physiological functions of melatonin are diverse and include the regulation of circadian rhythms, control of blood pressure and autonomic cardiovascular regulation, modulation of the immune system, regulation of energy expenditure and body mass, and vital roles in normal pregnancy and fetal development [25,26,115]. The principal mechanisms underlying melatonin's antioxidant properties involve scavenging ROS/RNS, promoting the expression of antioxidant enzymes (e.g., SOD and glutathione reductase), and increasing the availability of NO.

3.2. Melatonin in Pregnancy and Fetal Development

Melatonin assumes a crucial role in various stages of reproductive processes, encompassing ovulation, fertilization, embryo implantation, and acting as a regulator during pregnancy [116]. In pregnant women, nighttime blood melatonin concentrations are higher than those in their non-pregnant counterparts, peaking at term and returning to physiological levels post-delivery [116]. Maternal melatonin easily traverses the placenta, offering photoperiodic cues to the developing fetus [117]. Beyond the pineal gland, the placenta can independently generate melatonin in a circadian-independent manner [118]. This placental melatonin system plays a pivotal role in scavenging free radicals, thereby attenuating oxidative damage in compromised pregnancies [119]. Experimental evidence supports

melatonin's involvement in ensuring adequate placental perfusion, preventing vascular damage, local inflammation, and oxidative stress [116]. Conversely, a deficiency in maternal melatonin disrupts circadian rhythms, compromises organogenesis, and contributes to IUGR in adult rat offspring [119,120]. Additionally, melatonin exerts biological influence in timing the onset of spontaneous labor and the efficacy of uterine contractions during labor [116]. These findings underscore the indispensable role of melatonin in ensuring normal pregnancy and fetal development. Figure 3 encapsulates the role of melatonin in pregnancy.

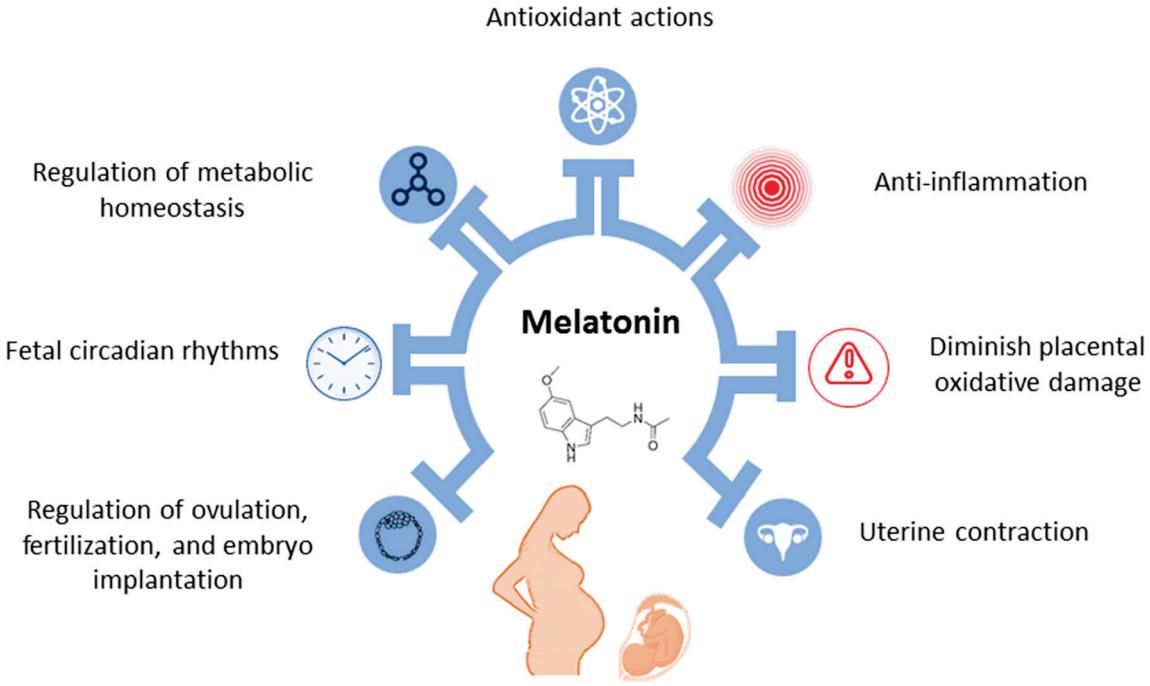


Figure 3. Role of melatonin in pregnancy.

3.3. Perinatal Melatonin Use in Animal Models of CKM Syndrome of Developmental Origins

Due to its antioxidant effects, melatonin shows potential efficacy in treating disorders associated with CKM syndrome, including obesity [121], diabetes mellitus [121], metabolic syndrome [122], CKD [123], hypertension [124], and CVD [125]. Although certain human studies have investigated the administration of melatonin during pregnancy and lactation, comprehensive reviews highlight the absence of focused assessments on the long-term outcomes for offspring [126–128].

To address this gap, Table 2 provides a summary of studies documenting the protective effects of melatonin as a reprogramming strategy in rat models related to CKM syndrome of developmental origins [78,81,129–139]. The therapeutic duration is limited to the perinatal period before the onset of disease. Table 2 includes various early-life insults that lead to offspring CKM syndrome, and perinatal melatonin use has been shown to avert these effects. These insults encompass maternal chronodisruption [77,78], maternal hypertension [129,138], maternal caloric restriction [130], maternal N^G-nitro-L-arginine-methyl ester (L-NAME) exposure [131], maternal high-methyl-donor diet [132], maternal high-fructose diet [133], maternal high-fat diet [134], perinatal glucocorticoid exposure [81,135–137], and maternal hypoxia [139]. Melatonin administration during pregnancy [129,139], lactation [137], or both periods [130–136] has demonstrated protective actions. Melatonin can be administered through injection [134,137] or via drinking water [78,129–136]. Table 2 showcases the protective effects of perinatal melatonin use in rat offspring aged from 3–27 weeks, corresponding to humans from childhood to young adulthood. In Table 2, the findings from animal studies suggest that melatonin doses within the range of 0.05–10 mg/kg/day demonstrate protective effects for rats.

Table 2. Protective effects of perinatal melatonin supplementation against CKM syndrome in rat models.

Treatment Period and Dose	Model	Age at Evaluation (Weeks)	Prevented CKM Syndrome in Offspring	Ref.
0.5 mg/kg/day p.o. throughout gestation and lactation	Maternal chronodisruption	18	Insulin resistance	[77]
10 mg/kg/day p.o. throughout gestation and lactation	Maternal chronodisruption	12	Hypertension	[78]
10 mg/kg/day p.o. during pregnancy	Maternal hypertension	8	Hypertension	[129]
10 mg/kg/day p.o. throughout gestation and lactation	Caloric restriction	12	Hypertension and kidney disease	[130]
10 mg/kg/day p.o. throughout gestation and lactation	Maternal L-NAME exposure	12	Hypertension and kidney disease	[131]
10 mg/kg/day p.o. throughout gestation and lactation	Maternal high methyl-donor diet	12	Hypertension	[132]
10 mg/kg/day p.o. throughout gestation and lactation	Maternal high-fructose diet	12	Hypertension	[133]
5 mg/kg/day i.p. throughout gestation and lactation	Maternal high-fat diet	3	Obesity, hyperglycemia, hyperlipidemia, and liver steatosis	[134]
10 mg/kg/day p.o. throughout gestation and lactation	Prenatal dexamethasone exposure	16	Hypertension, liver steatosis, and kidney disease	[81,135]
10 mg/kg/day p.o. throughout gestation and lactation	Neonatal dexamethasone exposure	16	Hypertension	[136]
1 mg/kg/day at night from postnatal day 2 to 14	Neonatal corticosterone exposure	16	Diabetes, hyperlipidemia, liver steatosis, and kidney disease	[137]
0.2 mg/kg/day p.o. throughout gestation and lactation	Maternal hypertension	27	Hypertension	[138]
0.05 mg/kg/day p.o.during gestation	Maternal hypoxia	16	Cardiovascular disease	[139]

p.o. = per oral; i.p. = intraperitoneal; L-NAME = N^G-nitro-l-arginine methyl ester.

3.4. Effects of Melatonin in Renal Programming

Accumulating evidence indicates that exposure to unfavorable environmental stimuli during kidney development raises the likelihood of CKD and hypertension in adulthood, primarily through renal programming [140,141]. Maternal melatonin deficiency has been implicated in causing renal programming, leading to offspring hypertension [142,143].

In a prior study, we scrutinized the renal transcriptome of male rat offspring born to dams receiving melatonin supplementation throughout pregnancy and lactation, covering the entire period of kidney development [144]. The results revealed 455, 230, and 132 differentially expressed genes in the offspring's kidneys at 1, 12, and 16 weeks of age, respectively. Maternal melatonin therapy was found to upregulate several epigenetic regulators during kidney development, indicating its potential epigenetic effects [145]. Furthermore, melatonin administration influenced numerous biological pathways associated with kidney development.

Being a widely acknowledged antioxidant, melatonin therapy has exhibited protective effects against oxidative-stress-related renal programming in adult rat offspring. This was observed in models of dams receiving a low-caloric diet [130], antenatal L-NAME administration [131], dams fed with diet rich in methyl donor [132] or fructose [133], and antenatal glucocorticoid administration [135]. Perinatal melatonin use also proved beneficial in terms of renal programming, particularly in rebalancing the ADMA–NO pathway. In a maternal caloric restriction model [130], the protective mechanisms of melatonin against offspring hypertension were associated with a reduction in plasma

ADMA, an increase in renal NO levels, and epigenetic changes in numerous genes within the offspring's kidneys.

3.5. Effects of Melatonin in Cardiovascular Programming

While the cardiovascular benefits of antioxidants, in general, await definitive confirmation [136], melatonin has emerged as a potential reprogramming intervention against cardiovascular programming complexities associated with maternal hypoxia [139]. Adult rat offspring exposed to hypoxic pregnancy displayed cardiac wall thinning and heightened vasoconstrictor reactivity, conditions mitigated by maternal treatment with melatonin. The protective effect of melatonin was linked to an increase in the cardiac protein expression of eNOS [139].

It is noteworthy that melatonin's cardioprotective attributes are also ascribed to its various antioxidant actions [146], including the inhibition of mitochondrial respiratory chain complex [147], the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [148], and the suppression of inflammatory cytokine release [149]. However, these mechanisms have yet to be thoroughly examined in animal models of oxidative-stress-related cardiovascular programming. A prior study suggested the beneficial role of melatonin in CVD programming in offspring primed by maternal diabetes [150]. While cardiac function and structure were comparable between adult mice offspring born to diabetic and nondiabetic dams, the diabetic offspring exhibited increased infarct size, cardiac dysfunction, and myocardial apoptosis in response to myocardial ischemia/reperfusion, along with heightened oxidative stress. Notably, maternal melatonin supplementation improved myocardial ischemic tolerance in the diabetic offspring.

3.6. Effects of Melatonin in Metabolic Programming

As outlined in Table 2, maternal melatonin therapy has demonstrated favorable effects against metabolic-programming-induced insulin resistance [77], obesity [134], hyperglycemia [134], hyperlipidemia [134,137], diabetes [137], and liver steatosis [134,135,137]. In a maternal high-fat diet model, maternal melatonin therapy shielded adult rat offspring from obesity, hyperglycemia, hyperlipidemia, and liver steatosis, concurrently reducing hepatic oxidative stress [134]. Another study indicated that liver steatosis induced by prenatal dexamethasone administration could be averted through maternal melatonin supplementation [135].

Adipose tissues are known to produce pro-inflammatory cytokines and ROS, which play a decisive role in the development of obesity [151]. Melatonin has the potential to counteract the detrimental effects of obesity on perivascular adipose tissue, addressing issues such as excessive ROS production, diminished SOD activity, and reduced NO availability [152]. Also, melatonin could stimulate the Nrf2 signaling pathway to reduce lipopolysaccharide-induced ROS generation [153] and ameliorate H_2O_2-induced oxidative stress through the modulation of the extracellular-signal-regulated kinase (ERK)/Akt/nuclear factor κ (NF-κB) pathway [154]. By activating antioxidant enzymes related to glutathione metabolism, melatonin could protect tissues such as the pancreas, adipose tissue, and liver from oxidative stress [151,155], through which it fights obesity-related disorders.

Similarly, in corticosterone-programmed diabetic offspring rats, melatonin demonstrated the ability to prevent metabolic programming [137]. The corticosterone-programmed diabetic offspring exhibited hyperglycemia, hypoinsulinemia, hyperlipidemia, and liver steatosis, accompanied by increased lipid peroxidation and decreased levels of enzymatic antioxidants. However, treatment with melatonin effectively prevented both CKM-syndrome-related components and oxidative stress [137].

3.7. Pros and Cons

Although animal studies provide evidence supporting melatonin as a promising reprogramming strategy for preventing CKM syndrome, its efficacy is yet to be validated

in human trials. While melatonin is classified as a dietary supplement in the United States, it is only available as a prescription drug in many countries [156].

Currently, oral melatonin supplementation in humans is considered to have a favorable safety profile, with typical doses ranging from 2 to 10 mg in various populations [30]. Experimental data have revealed that melatonin is effective as an antioxidant at high pharmacological doses. Notably, uncertainty surrounds the use, especially at high dosages, of melatonin by mothers during pregnancy and lactation. Clinical studies, thus far, do not recommend the use of melatonin in pregnant and lactating women [30].

In the rat models outlined in Table 2, melatonin administered at a dosage ranging from 0.05 to 10 mg/kg/day to dams demonstrates protective effects against CMK syndrome in their offspring. It is noteworthy that melatonin exhibits remarkable non-toxicity, with a high safety profile observed in rats. The lethal dose 50 for intraperitoneal melatonin injection in rats was determined to be 1168 mg/kg, while the oral administration of melatonin (tested up to 3200 mg/kg) could not be determined [157]. Calculating the human equivalent dose of melatonin for a 75 kg adult based on body surface area normalization from the doses used in various rat models yields a range of 0.6 to 120 mg [158]—approximately one order of magnitude higher than commonly employed doses in humans based on the experiments in Table 2 [159].

Despite these data, there remains a scarcity of information from clinical trials regarding the use and safety of melatonin in pregnant or breastfeeding women [128]. A phase I clinical trial involving 20 pregnant women with early-onset preeclampsia administered a daily total of 30 mg melatonin (in three 10 mg doses) and reported the safety of melatonin for both women and their fetuses, with no adverse events or reactions observed in mothers, fetuses, or neonates [160]. To the best of our knowledge, no trial results focusing on the safety or efficacy of melatonin during pregnancy and lactation specifically for offspring outcomes, especially related to CKM syndrome, have been published [161]. This review underscores the imperative need for well-powered clinical trials in this domain.

As reviewed elsewhere [29], melatonin has been investigated as a potential treatment for various neonatal conditions, encompassing periventricular leukomalacia [162], hypoxic–ischemic injury [163], respiratory distress syndrome [164], and sepsis [165]. Given the challenges of recruiting pregnant women for medical research, utilizing lactating women and their neonates as a reprogramming strategy presents a feasible starting point.

Another concern is that targeted oxidative stress therapy can potentially be harmful. Excessive antioxidant supplementation may shift oxidative stress to the opposite state, known as antioxidant stress [166,167]. Despite its antioxidant property, melatonin may exhibit prooxidant activity as a modulator of cellular redox status [168]. Additionally, healthy tissues not affected by oxidative stress damage may be non-specifically targeted by antioxidants. When melatonin circulates and reaches various organs, it may negatively affect healthy tissues by reducing their levels of ROS below physiologically normal limits. Given the importance of redox homeostasis for normal pregnancy and fetal development, melatonin supplementation as an antioxidant during pregnancy and breastfeeding should only be considered in cases of oxidative stress, not as a routine dietary supplement.

Melatonin operates through various mechanisms to exert its functions. The beneficial effects of perinatal melatonin therapy may be attributed to several mechanisms beyond antioxidants, which are known to interact with oxidative stress underlying CKM syndrome. However, these biological actions may also have negative consequences. In light of this, additional research is essential to determine whether any detrimental consequences emerge from the perinatal administration of melatonin to offspring.

4. Concluding Remarks

The ROS generated during fetal and perinatal stages have the potential to influence the long-term health of individuals, increasing their vulnerability to various adult diseases. The impact of oxidative stress extends to key organs such as the kidneys, heart, blood vessels, and metabolic control systems, eventually contributing to CKM syndrome. Despite

this understanding, a significant gap remains in animal models that directly explore the consequences of early-life oxidative stress on all aspects of CKM traits in adult offspring. Variations in outcomes may be attributed to diverse environmental insults, the susceptibility of specific developmental windows, organ-specific responses to oxidative stress, or the timing of offspring assessments. Consequently, further research is imperative to comprehend the intricacies of redox-sensitive signaling in different organs, which are affected by various maternal-derived insults during pregnancy and the perinatal period, influencing CKM programming. This knowledge will aid in the development of targeted antioxidant strategies to mitigate the impact of these insults on disease manifestation.

Additionally, melatonin emerges as a potential reprogramming strategy for preventing CKM syndrome. As a well-established antioxidant, melatonin exerts beneficial effects on human health, characterized by a favorable safety profile. Although animal studies showcase the notable advantages of melatonin therapy in CKM programming, its efficacy still awaits validation through human investigations. The anticipation is that melatonin may find application in clinical settings, influencing the interplay between oxidative stress and CKM syndrome in the future.

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Systematic Review

The Role of Xanthine Oxidase in Pregnancy Complications: A Systematic Review

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Abstract: Xanthine oxidoreductase (XOR) is an enzyme involved in the oxidation of hypoxanthine and xanthine to uric acid. XOR has two isoforms: xanthine dehydrogenase and xanthine oxidase (XO). XO plays a major role in oxidative stress, causing the formation of reactive oxygen species. In the present study, we aimed to summarize the evidence on the association between XO and pregnancy complications. The PRISMA checklist guided the reporting of the data. We conducted systematic searches in the PubMed and Web of Science databases to identify all human studies investigating XO in pregnancy diseases up to June 2024. A total of 195 references have been identified and 14 studies were included. Most studies focused on women with PE and GD. Overall, all the included studies found a statistically significant increase in maternal, placental, and/or fetal XO levels, activity, or tissue expression in women with pregnancy complications, compared to those with uncomplicated pregnancies. Although promising, the quality and dimension of the included studies do not allow for a definitive answer to the question of whether XO may play a crucial role in pregnancy complications. Future studies are warranted to confirm if XO could represent a prognostic and therapeutic marker in pregnancy complications and their impact on long-term maternal and offspring cardiovascular health.

Keywords: xanthine oxidoreductase; xanthine dehydrogenase; xanthine oxidase (XO); oxidative stress; placenta; pregnancy; pregnancy complications; preeclampsia; gestational diabetes

1. Introduction

Pregnancy is a metabolic state characterized by an elevated energy demand and an increase in oxygen consumption, which results in high mitochondrial activity and the production of many reactive oxygen species (ROS) in the placenta [1]. ROS are crucial signal transducers in physiological processes of normal placentation, including proliferation, migration, and angiogenesis, but ROS overproduction and the depletion of antioxidant systems can lead to oxidative stress (OS) and abnormal development of the placental vessels, leading to placental insufficiency [1–3]. ROS play a crucial role in cellular signaling, but their excess causes cellular damage, lipid peroxidation, DNA oxidation, protein modifications, and, consequently, cellular and tissue dysfunction [4]. Therefore, a dynamic balance between the generation of ROS and the actions of antioxidant systems (including enzymatic systems such as xanthine oxidoreductase (XOR), superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and catalase, and nonenzymatic antioxidants such as vitamin C and E, beta carotene, selenium, zinc, taurine, and manganese) is essential for maintaining ROS at optimal levels for proper cellular functioning [2,3]. XOR belongs to the highly conserved family of molybdo-flavoenzymes and performs the last two steps of purine catabolism [5]. XOR catalyzes the oxidation of hypoxanthine and xanthine to uric acid, which is the end product of purine catabolism in humans [6]. This enzyme has

two forms that coexist in vivo: xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XDH requires nicotinamide adenine dinucleotide (NAD+) and produces uric acid and reduced nicotinamide adenine dinucleotide (NADH). XO requires molecular oxygen as an electron acceptor and generates uric acid and ROS, namely superoxide ion ($O^{2\bullet-}$) and hydrogen peroxide (H_2O_2), if it catalyzes the monovalent or divalent electron transfer to O_2, respectively. Under physiological conditions, the enzyme XOR exists mostly as XDH and is located in the cytosol and peroxisomes, and is also found in extracellular compartments, such as blood and milk, as a result of physiological cell turnover and of the apocrine secretion from the breast during lactation [5]. Under certain conditions, including ischemia and hypoxia, the enzyme XOR is released from the cell into the bloodstream and the ATP depletion triggers the reversible conversion of the dehydrogenase form to XO through the oxidation of a sulfhydryl group. When reperfusion and re-exposure to normal oxygen tension occurs, the increased XO activity and expression results in excessive production of ROS [5,7] Therefore, XO plays an important role in the OS-related tissue injury in all the diseases characterized by ischemia–reperfusion (H/R) conditions, including myocardial infarction, stroke, acute renal failure, and diseases affecting the maternal–fetal unit [7,8]. Indeed, XO may play a key role in many obstetric conditions characterized by altered placentation due to but not limited to insufficient trophoblastic remodeling of uterine spiral arteries and consequent increase in placental vascular resistance. The increased resistance and altered flow lead to an ischemic injury at the intervillous space that could trigger the conversion of XOR to XO, with increased OS contributing to the altered maternal adaptation to pregnancy and the onset of pregnancy-related pathological conditions [9]. XOR also plays a central role in redox homeostasis and adaptation to pregnancy by regulating uric acid levels. Indeed, uric acid is among the main antioxidant systems, accounting for 50% of the total antioxidant capacity of biological fluids in humans while, when present in the cytosol of the cells or an acid/hydrophobic environment, turns into a pro-oxidant agent, promoting OS [10]. Uric acid can scavenge oxygen radicals and peroxyl radicals in the hydrophilic environment, such us plasma, but loses the ability to break the radical chain propagation and can also form free cytotoxic radicals, such as aminocarbonyl, in a variety of systems within lipid membranes. Therefore, the double oxidant and antioxidant function of uric acid plays a key role in the redox homeostatic system, and an imbalance towards excess uric acid represents a pathophysiological mechanism in the onset of many diseases [11]. ROS-induced OS not only leads to important pregnancy diseases such as preeclampsia (PE) and gestational diabetes (GD) but could also alter fetal development [3]. Furthermore, OS increases the risk for the development of several chronic diseases later in the life of the offspring (including ischemic heart disease and type 2 diabetes mellitus) through epigenetic modifications due to harmful uterine-environmental exposures. On the other hand, in the mothers, excessive OS may influence fertility, by inducing apoptosis in oocytes and interfering with chromosomal disjunction during meiosis, although the presence of ROS is needed for hormone production and ovulation [9]. Prolonged exposure to high ROS concentration also contributes to a wide range of pathologies, such as cancers, cardiovascular diseases, and neurological diseases [12].

Major pregnancy complications are associated with an increased long-term cardiovascular risk for both the mother and the fetus; for instance, PE determines a 4-fold increased risk of future heart failure and a 2-fold increased risk of coronary heart disease and stroke [13], whereas GD is responsible for a 2.3-fold increased risk of cardiovascular events in the first decade postpartum [14]. These associations may be partly explained by XO-induced OS. Indeed, studies of large cohorts of patients have demonstrated that XO is independently associated with cardiovascular risk due to the detrimental systemic effects of OS. Excessive XO activity contributes to inflammatory responses, the oxidation of low-density lipoprotein particles, and the formation of advanced glycation end products, all associated with the atherosclerotic process and the development of major cardiovascular events. XO-derived OS may also contribute to the progression of cardiovascular disease-associated endothelial dysfunction since superoxide radicals inactivate endothelial

NO, an important vasodilator agent [15–17]. Finally, the terminal product of the reaction catalyzed by XO is uric acid, which is increased both in some pregnancy pathologies and in cardiovascular disease [18,19].

In summary, XOR, and particularly, XO are involved in multiple pathophysiological mechanisms, such as OS, hyperuricemia, and H/R lesions, that, in turn, are associated with short- and long-term detrimental effects of pregnancy complications in both the mothers and their offspring. In the current study, we aimed to systematically review current evidence on the role of XO in pregnancy complications.

Although OS is a recognized mechanism contributing to preterm birth (PTB) and recurrent pregnancy loss (RPL), no studies investigating XO as a marker of OS in the context of RPL and PTB were identified. Therefore, we focused on the role of XO in HDP (HDP), GD, and intrauterine growth restriction (IUGR).

2. Materials and Methods

This systematic review did not require ethics approval because it involved completed research findings. The PRISMA checklist guided the reporting of this systematic review. We performed a systematic search using the electronic databases PUBMED and Web of Science. Two independent reviewers screened the articles and evaluated the quality of the included studies. Disagreements have been resolved by consensus or, when an agreement was not reached, with the help of a third reviewer.

2.1. Protocol

This systematic review was conducted according to the last Preferred Reporting Items for Systematic Reviews and Meta-analyses protocols (PRISMA-P) guidelines [20]. The systematic review was initially conducted as part of an internal project within the institution, and there was no requirement to register the protocol. To ensure transparency in the process, we intended to register the protocol, but data extraction and analysis were already completed.

2.2. Eligibility Criteria

The literature search was limited to the English language and human subjects. We excluded reviews and we included cross-sectional studies, case–control studies, cohort studies, case reports, case series, and histological studies. Exclusion criteria were as follows: publications evaluating markers of OS that have not assessed XO levels, activity, or expression; publications concerning the assessment of XO in diseases other than pregnancy complications; animal studies; and publications not in English. The eligible studies should include at least one of the following outcomes: miscarriage, IUGR, PE, gestational hypertension, premature delivery, and GD. Publications were included if maternal serum or cord blood XO levels, maternal blood, cord blood or placental XO activity, or XO expression in placental, or other tissue samples were assessed. No restrictions were made regarding the comparison groups or the XO measurement methods.

2.3. Information Sources and Search Terms

A systematic literature search was performed in the following databases from inception until 31 July 2024: Pubmed and Web of Science Core Collection.

Search terms used in the current search included: “Pregnancy”, “Xanthine oxidoreductase”, “Xanthine dehydrogenase”, “Xanthine oxidase”, “oxidative stress”, “placenta”, “pregnancy”, “early miscarriage”, “intrauterine growth restriction”, “preeclampsia”, “gestational hypertension”, “premature delivery”, and “gestational diabetes”.

2.4. Study Selection, Data Extraction and Quality Assessment

All articles were imported into Zotero. After eliminating duplicates, two reviewers independently screened the abstracts and full texts of the records to assess eligibility.

Detailed descriptions of the participants and interventions in each study are provided in the text and summarized in Table 1.

Table 1. Table summarizing the content of the 14 included studies.

Author, Year	Studied Population	Methods of XO Measurement	Results
A. Many et al., 2000 [21]	Histological study: 12 control placental specimens obtained from HPC vs. 10 placental specimens obtained from PE women.	Examination of XDH/XO holoenzyme activity and XO isoenzyme activity in placental villi and placental bed curettings by radiochemical method, adding xanthine and evaluating NAD production. Expression of XDH/XO in tissue samples by immunohistochemical staining.	In control samples, no XO expression in chorionic villi was found; a relatively weak XO immunoreactivity was found only in syncytiotrophoblasts. In PE samples, immunoreactivity dramatically increased, associated not only with the trophoblast, but also with the stromal cores of the blood vessels. Also, invasive cytotrophoblasts manifest increased expression of XO enzyme in PE. No difference in XDH/XO activity and XO activity was detected in placental villi. In placental bed curettings XO activity was significantly higher in the PE group compared with the control group.
Ilona Németh et al., 2002 [22]	Case–control study: 16 pregnant women with gestational hypertension vs. 14 HPC (matched for maternal age, parity, and gestational age) vs. 15 HC (matched for age)	After administration of a dose of caffeine (10 mg/kg), XO activity was estimated as ratio of two urinary metabolites of caffeine, 1 MX, the substrate of XO, and 1 MU, the product of XO measured by high-pressure liquid chromatographic method. Urine was collected over a 6 h period after caffeine administration.	A significant decrease in the urinary concentration of 1 MX was shown in the hypertensive subjects without any decrease in the urinary concentration of 1 MU. As a result, the XO activity index rose significantly in this group. Increased levels of UA were shown in hypertensive individuals. Other markers of OS, such as lipid peroxidation products, were more elevated in hypertensive patients compared to both HC and HPC. The XO activity index was higher in pregnant women with hypertension (0.849 ± 0.096) than in HPC in ed HC (0.596 ± 0.105 $p < 0.01$; 0.542 ± 0.049 $p < 0.01$, respectively).
Abdulkadir Yildirim et al., 2004 [23]	Case–control study: 25 PE women (17 mild PE and 8 severe PE) vs. 15 HPC vs. 15 HC.	Plasma XO activity was assayed spectrophotometrically.	Mean plasma XO activity was higher in both mild ($p < 0.05$) and severe ($p < 0.001$) PE groups than in the HPC group, with PE severity defined by blood pressure values. Other markers of OS showed a reduction in antioxidant systems (SOD, GSH-Px) in PE compared to both HPC and HC groups. In the PE group, SBP and DBP had a significant positive correlation with plasma XO activity ($r = 0.59$, $p < 0.01$ and $r = 0.68$, $p < 0.001$, respectively).
Aysun Bay Karabulut et al., 2005 [24]	Case–control study: 29 PE singleton pregnancies vs. 33 HPC (matched for maternal age and gestational age).	Plasma XO activity was assessed in maternal and cord blood by spectrophotometric methods. Maternal blood was drawn from the antecubital vein immediately after delivery.	XO and ADA activities and MDA levels in maternal and fetal plasma were significantly higher in the PE group than in the controls ($p < 0.05$).
A. Biri et al., 2006 [25]	Case–control study: 13 singleton pregnancies complicated by GD vs. 13 singleton HPC (matched on gestational age, maternal age, and BMI).	XO assessment was performed In maternal and cord plasma, and placental tissue samples (both collected at the time of Cesarean section). XO activity was analyzed spectrophotometrically.	XO activity of the GD group was significantly higher in maternal plasma ($p < 0.05$), cord plasma ($p < 0.05$), and placental tissues ($p < 0.005$), compared to the control group. Higher levels of HbA1c were correlated with lower levels of AOP ($r = -0.419$; $p < 0.05$) and MDA ($r = -0.348$; $p < 0.05$) in cord blood, and higher levels of MDA in maternal serum ($r = 0.405$; $p < 0.05$), which means that OS in maternal blood is associated with diabetes progression.
A. Biri et al., 2007 [26]	Case–control study: 13 pregnancies complicated by IUGR vs. 12 singleton HPC (matched on gestational age, maternal age, and BMI). The fetuses with a fetal birthweight < 10th percentile for gestational age were defined as having IUGR.	In the cord and maternal plasma and placenta tissue samples (both collected at the time of Cesarean section) XO activity was assayed spectrophotometrically.	Maternal serum XO activity was significantly higher in the IUGR group than in the control group (1.251 ± 0.674 mIU/mL vs. 0.20 ± 0.019 mIU/mL; $p < 0.0005$). Cord plasma levels of XO were also significantly higher in the IUGR group (1.97 ± 0.73 mIU/mL vs. 0.237 ± 0.143; mIU/mL, $p < 0.0005$). Placental levels of XO were significantly higher in the IUGR group than in the control group (0.023 ± 0.001 mIU/mL vs. 0.12 ± 0.004 mIU/mL; $p < 0.025$). Other redox metabolites showed a similar trend between the two groups, except for plasma levels of CAT. Placental tissue XO ($p < 0.005$) and other markers levels were significantly higher, while the CAT level ($p < 0.005$) was lower in the IUGR group.

Table 1. *Cont.*

Author, Year	Studied Population	Methods of XO Measurement	Results
Shannon A. Bainbridge et al., 2009 [27]	Case–control study: 5 PE vs. 5 HPC (matched for age, pre-pregnancy BMI) vs. 6 HC vs. 15 nonpregnant with inflammatory disease (5 with systemic lupus erythematosus, 3 with dermatitis, 1 with lichen simplex, 1 with mixed connective tissue disease, 5 with bullous pemphigoid).	Expression of XO was assessed by immunohistochemical fluorescent staining of skin biopsies. In all the pregnant women, biopsies were obtained from the cesarean section skin incision. In the nonpregnant participants with chronic inflammatory disorders, the biopsies were collected from lesional skin. In the HC, the skin biopsies were collected from benign skin lesions.	PE skin biopsies demonstrated intense XO immunoreactivity within the stratum granulosum layer of the epidermis. Faint and sporadic XO-positive staining was observed in the stratum granulosum layer of the epidermis in all the healthy pregnant control biopsies. All skin biopsies collected from nonpregnant patients with inflammatory conditions demonstrated immunoreactivity to XO within the stratum granulosum layer of the epidermis. Skin biopsies collected from HC demonstrated very little to no XO immunoreactivity.
M. Bogavac et al., 2012 [28]	Case–control study: 95 women with complicated pregnancy (54 with local infection, 18 GD, and 23 gestational hypertension) vs. 66 HPC.	Levels of XO were determined spectrophotometrically in amniotic fluid samples collected between 16 and 19 weeks of pregnancy during amniocentesis.	XO activity, though very low, was present in amniotic fluid samples and there was a statistically significant difference in XO activity between controls and all investigated study subgroups: patients with bacterial vaginosis (p-value < 0.015), patients with PIH (p-value < 0.001), and patients with GD ($p < 0.001$). Interestingly, other antioxidant enzyme systems showed no differences between groups.
V. Bambrana et al., 2015 [29]	Case–control study: 50 PE pregnancies vs. 50 HPC.	Plasma XO activity (measured with the spectrophotometric method was determined from samples during antenatal (at 30–39 weeks of gestation) and postpartum period.	The plasma XO activity was elevated in the PE group compared to HPC before (205 U/L ± 197.02; 39.10 U/L ± 54.04, respectively, $p < 0.001$) and after delivery (96.6 ± 141.3 U/L vs. 17.9 U/L ± 14.2; $p < 0.001$). The plasma XO activity registered during gestation decreased after delivery in both groups. Differences in NO were not significant. Pregnancy levels of SUA were higher in PE group.
Min Shang et al., 2015 [30]	A case–control study: 28 singleton GD vs. 40 HPC matched on gestational and maternal age, BMI, and parity.	Plasma XO levels were determined in maternal and cord plasma and placenta samples by spectrophotometric method.	The maternal, cord, and placental XO was significantly higher in GD women compared to HPC (p-value < 0.05). Other oxidative markers showed similar trends. Maternal plasma XO levels of GD vs. HPC were 15.75 ± 1.65 nmol/mL vs. 8.86 ± 1.97 nmol/mL, $p < 0.05$. Cord plasma XO levels were 14.13 ± 1.78 vs. 9.92 ± 2.20 nmol/mL, $p < 0.05$. Placental XO levels 51.18 ± 3.43 nmol/mL vs. 43.47 ± 8.02 nmol/mL, $p < 0.05$. Cord and placental XO had a significantly positive correlation with HbA1c values (R = 0.43 and R = 0.58, respectively). Several antioxidant status markers had a significant negative correlation with HbA1c values ($p < 0.05$). Increased XO levels in cord plasma were also found in macrosomia ($p < 0.05$).
Oguz Elmas et al., 2016 [31]	Case–control study: 20 PE pregnancies vs. 20 HPC (matched on gestational age and maternal age).	Plasma XO activity was assessed by high-performance liquid chromatography.	Serum XO activity was significantly higher in PE groups than in controls (0.49 ± 0.22 microM/min/L vs. 0.25 ± 0.13 microM/min/L; $p < 0.0001$). The XO activity, UA, and allantoin levels showed high correlations with blood pressure in the PE group (but not in the controls). Correlation between XO activity and blood pressure in PE group: r = 0.47 $p = 0.039$ for DBP; r = 0.37 $p = 0.11$ for SBP; r = 0.47 $p = 0.038$ for MAP. There was no statistically significant difference between XO activity and UA in terms of prediction of PE. However, XO activity had the highest predictive value. No differences were observed between groups in nitrite levels and their relationship with blood pressure values.
Ebru Biberoglu et al., 2016 [32]	Case–control study: 20 singleton pregnancies with an IUGR fetus vs. 20 singleton HPC (matched for mean age, BMI, and weight gain during pregnancy).	XO activity was measured spectrophotometrically in the myometrial lysates and maternal serum samples.	Serum XO was higher in IUGR vs. HPC (0.21 ± 0.04 mIU/L vs. 0.19 ± 0.02 mIU/L, $p = 0.11$), and myometrial XO lower (0.012 ± 0.01 mIU/mg; 0.013 ± 0.001 mIU/mg, $p = 0.24$), although differences were comparable. MDA and CAT concentrations were higher in the serum ($p < 0.05$) but lower in the myometrial samples ($p < 0.01$) of women with IUGR than HPC.

Table 1. *Cont.*

Author, Year	Studied Population	Methods of XO Measurement	Results
Min Shang et al., 2018 [33]	A case–control study: 105 pregnancies with GD vs. 103 HPC.	XO was measured in maternal plasma, cord plasma, and placenta samples with commercial kits by colorimetric methods using a spectrophotometer.	XO levels in maternal and cord plasma and placenta samples were significantly higher in women with GD ($p < 0.05$) compared to controls. The levels of markers of OS were altogether positively correlated with HOMA Index in maternal plasma, cord plasma, and placenta (r = 681 $p < 0.001$; r = 651 $p < 0.001$; r = 525 $p = 0.072$, respectively), whereas they were negatively correlated with QUICKI (r = −0.761 $p < 0.001$; r = −0.720 $p < 0.001$; r = −0.533 $p < 0.001$, respectively). All of the studied markers of OS in the macrosomic newborns were higher than in normal birthweight newborns and were negatively correlated with birthweight ($p < 0.05$).
Ramya Rajshekar et al., 2021 [34]	Case–control study: 30 women with PE Vs. 30 HPC matched for maternal age.	Plasma levels of XO were estimated spectrophotometrically.	XO level was significantly more elevated in PE (218.78 ± 220.42 U/L) than in controls (34.01 ± 38.26 U/L), $p < 0.001$. XO levels were positively correlated with elastase levels (r = 0.320; $p < 0.05$). Elastase was 4.5-fold more elevated in PE patients (26.81 ± 77.95 U/mL) than in controls (6.02 ± 3.4 U/mL), but the difference was not statistically significant ($p > 0.05$).

According to STROBE criteria, 5 articles obtained a score of 20 over 22 [22,25,27,30,34], 6 articles obtained a score of 19 over 22 [21,23,26,31,33], 1 article was scored 18/22 [24], 2 obtained a score of 16 over 22 [28,29]. List of abbreviations: ADA, Adenosine deaminase; AOP, antioxidant potential; BMI, Body Mass Index; CAT, catalase; DBP, Diastolic Blood Pressure; GD, gestational diabetes; GSH-Px, glutathione peroxidase; GSHR, glutathione reductase; GST, glutathione S-transferase; HC, healthy nonpregnant controls; HOMA-IR, homeostasis model assessment of insulin resistance; HPC, Healthy pregnant controls; HX, Hypoxanthine; IUGR, intrauterine growth restriction; LPO, lipid peroxides; MAP, mean blood pressure; MDA, malondialdehyde; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; SBP, Systolic Blood Pressure; PE, preeclampsia; QUICKI, quantitative insulin sensitivity check index; ROC, receiver operating characteristic; SOD, superoxide dismutase; TAC, total antioxidant capacity; UA, uric acid; X, xanthine; XDH, xanthine dehydrogenase; XO, xanthine oxidase; 1 MX, 1-methylxanthine; 1 MU, 1-methyluric acid; 8IsoP, 8-isoprostane.

The methodological quality and risk of bias of the selected studies were assessed using the STROBE standard [35], specifically by applying the 22-item STROBE checklist. Two authors independently performed the scoring, with any disagreements resolved through discussion and consensus. When agreement was not reached, a third author was consulted. Studies meeting 10 to 16 out of 22 criteria were classified as having medium quality and medium risk of bias; those exceeding or falling below this range were classified as high-quality and low-quality, respectively. The overall quality of the included studies was moderate to high, and the number of cases was low to moderate (the number of enrolled women, both cases and controls, was <50 in 7 studies, 50–99 in 4 studies, and 100–200 in 3 studies).

3. Results

A total of 195 references were identified (107 from Pubmed and 88 from Web of Science). After removing duplicated articles (n = 38), 157 articles were screened. In total, 109 screened articles were excluded: 14 studies were removed because they were secondary studies and 95 studies were removed for not assessing XO with any kind of method. A total of 48 articles were eligible for full-text reading; 34 records of these were excluded from this systematic review for not assessing the relationship between XO and pregnancy complications listed in the eligibility criteria. In total, 14 articles fulfilled the inclusion criteria (Figure 1). No articles were found for the following outcomes: early miscarriage and premature delivery.

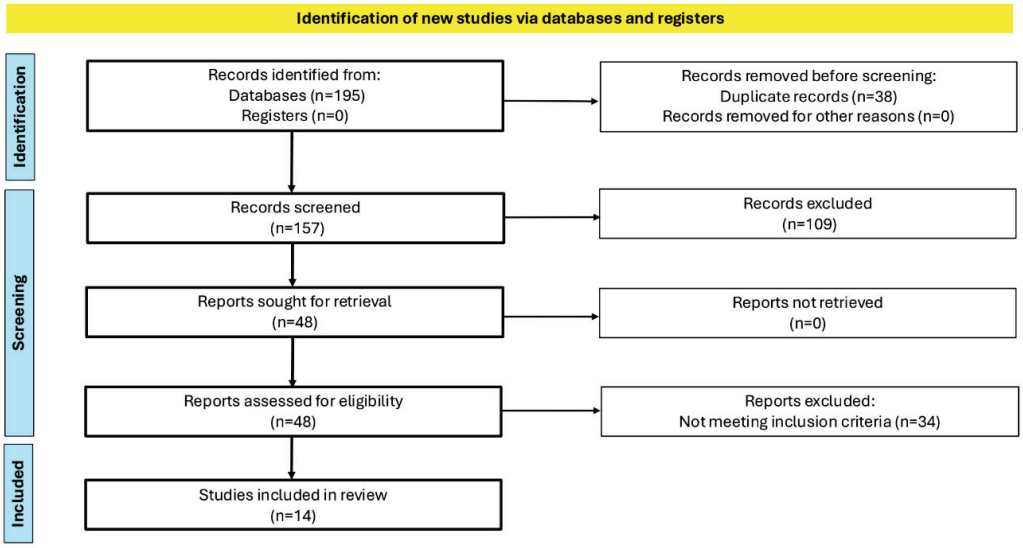


Figure 1. Flow diagram illustrating the article selection process of this review. XO: xanthine oxidase.

4. Discussion

Figure 2 summarizes the metabolism of purines up to the formation of uric acid (terminal product in humans) and highlights the formation of superoxide radicals in the reaction catalyzed by XO. In conditions of ischemia, the conversion of XDH to XO promotes OS and, consequently, damages at the subcellular, cellular, and tissue levels. In the upcoming text, we aim to provide a summary of the current literature on the role of XO activity and expression, both at the placental and systemic levels, in the main complications of pregnancy.

XO-induced and XO-independent ROS are crucial signal transducers in the physiological processes of normal placentation, including proliferation, migration, and angiogenesis. Appropriate development of the placental vascular network requires vasculogenesis, angiogenesis, and trophoblast-mediated arterial remodeling [2]. In the first trimester, the embryo develops in a low-oxygen environment, allowing the proliferation of trophoblastic cells [36]. The trophoblastic invasion of uterine arteries converts the spiral arteries into a chamber of high flow and low velocity, resulting in higher oxygen tension in the intervillous space. At the beginning of the second trimester, the increase in oxygen tension and the high energy demand lead to increased mitochondrial activity, resulting in intrauterine mild ROS production, as a physiological response to the pregnancy [1]. As shown in Figure 2, when the production of ROS during pregnancy is not balanced by antioxidant defenses, the consequential OS could disturb fetal development and increase the risk for IUGR, PE, premature delivery, and GD [9]. Therefore, reducing OS during gestation may represent a therapeutic target to improve pregnancy outcomes. OS can be measured in three major ways: the direct measurement of ROS levels; the indirect measurement of ROS-induced protein, lipid, and DNA damage or ROS-producing enzymes; and the assessment of antioxidant status [37]. However, there is no consensus on which marker is the best in various obstetric conditions [38]. Assessing XO activity has advantages over other OS markers; it is involved in multiple pathophysiologic pathways and could provide an estimate of future cardiovascular risk since increased XO levels characterize both pathological pregnancies and cardiovascular diseases [13,14,17]. Furthermore, since XO inhibitors exert cardioprotective effects in patients with symptomatic hyperuricemia, they may exert similar benefits in women with a previous history of pregnancy complications [39]. Furthermore, the use of XO inhibitors has shown potential in reducing fetal brain damage due to asphyxia [40–42].

Before discussing the role of XO in specific obstetric pathological conditions, in the following, we summarize the general aspects of XO activity in humans, including the biochemical mechanisms underlying XO-induced ROS production, the methods used to assess XO activity, and the pathophysiological effects of XO activity dysregulation.

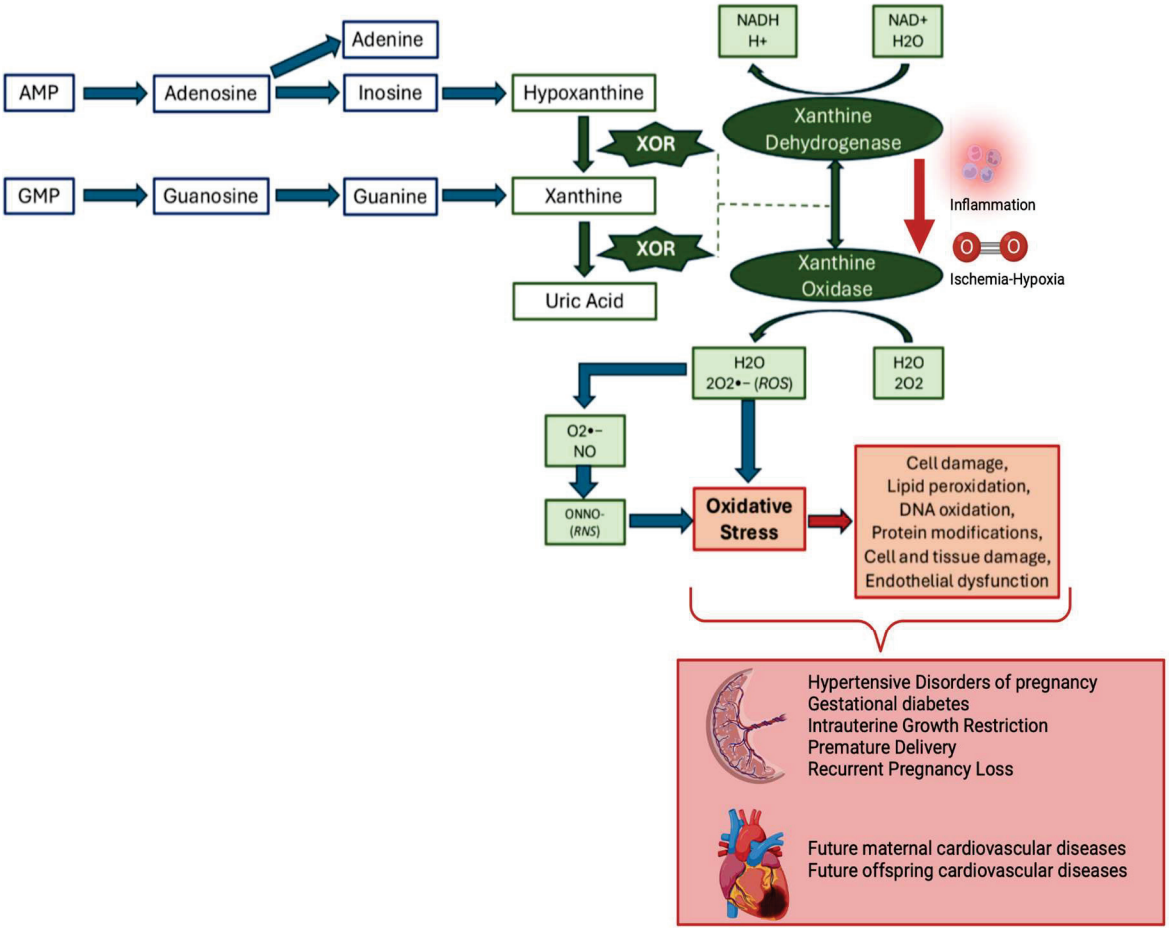


Figure 2. Catabolism of purines up to the formation of uric acid by the two isoforms of the enzyme XOR: xanthine dehydrogenase and xanthine oxidase. In case of ischemia, hypoxia, or inflammation, the conversion of the xanthine dehydrogenase into the xanthine oxidase isoform causes the excessive formation of superoxide radicals that increase OS. Furthermore, the superoxide radical ($O^{2-\bullet}$) reacts with the nitric oxide produced by the endothelium, determining the formation of peroxynitrite (ONNO-), which also contributes to OS. Then, OS causes damage at the subcellular, cellular, and tissue levels, inducing pregnancy complications and long-term cardiovascular dysfunction in both the mother and the offspring. List of abbreviations: OS, oxidative stress; XOR, xanthine oxidoreductase; ROS, reactive oxygen species; RNS, reactive nitrogen species. Image created with BioRender.com.

4.1. History, Molecular Characteristics, and Tissue Expression of XOR

XOR is a dimeric metallo-flavoprotein enzyme that catalyzes the final two steps of purine degradation: the oxidation of hypoxanthine and xanthine to uric acid. This enzyme consists of two subunits, each of 145 kDa: one contains a molybdopterin cofactor (Mo-co), where purine oxidation occurs, and the other contains a FAD cofactor, which is required for the oxidation of nicotinamide adenine dinucleotide (NAD+) and the reduction in molecular oxygen [43]. After human XDH cDNA was cloned and sequenced, and the relevant gene was located on chromosome 2 at the p22 band using the FISH method (fluorescence in situ hybridization) in 1994, studies were conducted to determine which tissue exhibited the highest enzyme expression [44,45]. A study published in 1998 quantified XOR gene expression by measuring the amount of RNA in tissue samples using RT-PCR (Reverse Transcriptase Polymerase Chain Reaction). XOR mRNA expression was found to be highest in the gut and liver, while the cardiac muscle and brain showed the lowest expression levels [7]. XOR is also detectable in other tissues and cell types, such as the kidney, lactating mammary gland, epithelial cells, and vascular endothelial cells. In many other tissues, XOR gene expression is low due to downregulation. Indeed, although XOR is present in

all human tissues, in most cases the XOR gene is typically repressed at the transcriptional level [43]. However, various factors can upregulate XOR gene transcription, including hypoxia, inflammatory cytokines, and certain hormones [46]. For example, XOR expression increases during pregnancy due to exposure to glucocorticoids and prolactin [47].

The history of the discovery of XOR at the molecular and genetic levels, along with a more in-depth discussion of the tissues that express XOR, is summarized in the review by Kooij et al., 1994 [48]. The first studies on placental expression of XOR, conducted around the 1980s, reported the virtual absence of enzyme activity in fresh human placenta, measured spectrophotometrically [49]. However, using more sensitive laboratory methods, Many et al. in 1996 were the first to demonstrate the presence of XOR in the human placenta. They detected XDH/XO-RNA using Northern hybridization, and XOR tissue expression by immunohistochemistry both in villous and non-villous trophoblast cells. Furthermore, XOR enzyme activity was also detected through the radiochemical method, although XDH/XO activity in the placenta was much lower than in the liver [50].

4.2. Methods of Assessment of XO Activity

It is important to note that there are various methods to assess XDH/XO activity in bio samples, and not all the approaches have the same sensitivity, such as spectrophotometry, fluorometry (FL), colorimetry, and radiometry [51]. The radiochemical assay is 10 times more sensitive than the spectrophotometric assay. The radiochemical assay uses a radioactive marker to evaluate the conversion of [14C] xanthine or [14C] hypoxanthine (contained in the reaction mixture) to [14C] uric acid, while the spectrophotometric assay measures the formation of uric acid from xanthine [50,51]. Several studies also employ a combination of high-performance liquid chromatography and ultraviolet detection (LC/UV). This method uses xanthine as a substrate; LC allows the separation of the produced uric acid, while UV detectors measure UA production. Other variants include LC/FL, which utilizes fluorescence detection, and LC/HRMS (high-resolution mass spectrometry), which uses mass spectrometry [51]. A study conducted at the University of Bologna by Battelli et al. recommends performing the evaluation of XDH/XO enzymatic activity using an ELISA method, demonstrating that it is more sensitive than the spectrophotometric method and easier to be implemented in routine clinical analyses compared to other sensitive tests (radiochemical, high-performance liquid chromatography, or FL methods). However, ELISA assays compared to enzyme activity-based assays, detect both active and inactive enzyme molecules [52]. Immunohistochemistry and XO plasma concentrations can help identify the level of XO expression in tissue but they do not provide precise information on XO activity since they detect both the active and inactive forms of the enzyme. For this reason, 12 out of the 14 included studies evaluated XO activity, while 1 study (Shannon A. Bainbridge et al., 2009 [27]) assessed only immunohistochemical expression, and 1 study (Ramya Rajshekar et al., 2021 [34]) measured only XO plasma levels.

Most of the studies included in the systematic review evaluating XO activity used the spectrophotometric method. Only the study by Oguz Elmas et al. [31] used a more sensitive method, namely high-performance LC, whereas another included study indirectly estimated XO activity as the ratio of two urinary metabolites of caffeine (1 MX, the substrate of XO, and 1 MU, the product of XO), measured with the high-pressure LC method in urine samples after caffeine administration. As XO is also involved in the metabolism of methylxanthines, the caffeine metabolic ratio 1 MU/(1 MX + 1 MU) has been shown to be a specific indicator of in vivo XO activity [53]. Overall, XO activity was assessed in maternal serum, cord blood, and placental samples (after homogenization and centrifugation). Furthermore, we emphasize that only one study compared the proportions of the enzyme in its different isoforms by evaluating [14C] uric acid production in the presence (XDH) and absence (XO) of NAD (A. Many et al., 2000 [21]). All other studies evaluated only XO, as it is the XOR isoform that primarily contributes to ROS production, promoting OS and, consequently, the development of numerous pathological conditions.

4.3. Role of XO in ROS Production: The Ischemia Reperfusion Mechanism

Although XOR is constitutively present in its dehydrogenase form, various conditions can promote the reversible conversion of NAD+-dependent XDH into the oxidase form through sulfhydryl group oxidation, or irreversibly through limited proteolysis [54]. In particular, many studies have focused on the role of the ischemia–reperfusion (H/R) mechanism in increasing XO activity and expression. Ischemia first induces Ca++-dependent proteolytic conversion of XDH to XO and increases levels of its substrates (xanthine and hypoxanthine). Then, reperfusion provides oxygen to the XO enzyme, leading to ROS production, which causes cytotoxicity and tissue damage [55]. In the reaction catalyzed by the oxidase form, oxygen acts as the electron acceptor, producing superoxide ions through a one-electron reduction and generating hydrogen peroxide via a two-electron reduction [43]. Moreover, hypoxia and inflammatory cytokines (TNF-α, IL-1β, IFN-γ) induce XDH expression in tissues and vascular endothelial cells, from which it is released into the circulation. Circulating XDH is quickly converted to XO, which then avidly binds to the negatively charged glycosaminoglycans (GAGs) on the apical surface of vascular endothelial cells, amplifying local ROS concentrations [56]. Oxygen tension influences XOR activity not only by regulating the gene expression of the enzyme but also through post-translational regulation, including phosphorylation and sulfurization of XOR molecules. For instance, ischemia increases mRNA expression (leading to an increased amount of XOR proteins) via pre-translational regulation, while also boosting XO activity by promoting the phosphorylation of XOR molecules.

It is also worth mentioning that, contrary to previous beliefs, the XDH isoform can also produce ROS. When NAD+ is abundantly available, the generation of O^{2-} by XDH is limited. However, under ischemic conditions, XDH operates as an oxidase of NADH and generates superoxide radicals by transferring an electron from NADH to FAD and reducing molecular oxygen. Nevertheless, our review has focused on the role of XO in ROS production and tissue damage [43].

4.4. The Ischemia Reperfusion Mechanism: Human Placental In Vitro Studies

OS is implicated in the pathogenesis of various obstetric pathologies, including HDP [57–62], early miscarriage [60,62,63], GD [64–66], preterm delivery [67], and fetal growth restriction [68]. Several in vitro studies have been conducted to determine if the H/R pathway could be one of the mechanisms responsible for the increase in OS observed in diseased placental tissue. It has been shown that several changes observed in placental tissue during in vitro altered perfusion are similar to those reported in placentae from certain pregnancy pathologies, suggesting that H/R may be one of the underlying pathophysiological mechanisms of many obstetric conditions. In 2001, Hung T. and colleagues examined the oxidative status of human placental samples in vitro during periods of hypoxia and reoxygenation (H/R model). Using a fluorogenic probe, they detected a high generation of ROS in the villous endothelium and, to a lesser extent, in the syncytiotrophoblast and stromal cells when hypoxic tissues were reoxygenated [69]. The resulting OS stimulated apoptosis in human placental tissues since H/R could induce the release of cytochrome c from mitochondria and the activation of caspases in the syncytiotrophoblast and fetal endothelial cells [70]. Another in vitro model that allows for the analysis of H/R-induced increase in OS is the dual-perfused placental tissue model. This model compares the perfusion of an isolated cotyledon of term placenta using a standard medium and a medium containing xanthine plus XO, which generates ROS [71]. This model demonstrated that the inflammatory response following exposure to the X + XO medium is responsible for the immunohistochemical staining of IL-1b in the villous stromal cells, an increase in various inflammatory cytokines (TNF-alpha, IL-1beta, IL-6, IL-8, and IL-10) in the maternal compartment, accumulation of 8-isoPGF2alpha (a marker of lipid peroxidation) in the fetal compartment, and shedding in the maternal compartment, as shown via flow cytometry [71]. Additionally, a study conducted by Murata et al. demonstrated that incubating a cell culture of extravillous trophoblast (EVT) cells with X + XO induced ROS production

by XO, which in turn triggered apoptosis and altered EVT functions, including invasion, tube-like formation, and differentiation [72].

4.5. Role of XO in Obstetric Pathologies: Discussion of Studies Included in the Review

After clarifying the role of XO and ROS production in the placenta, as well as the association between OS and placental diseases, we now delve deeper into the specific role of XO in individual obstetric pathologies by analyzing the studies included in this systematic review.

4.5.1. XO and Hypertensive Disorders of Pregnancy

Hypertensive disorders, including PE and gestational hypertension, are common pregnancy complications and represent major causes of maternal and fetal morbidity and mortality worldwide [73]. Pathogenetically, the historical hypothesis sees various pathogenetic mechanisms that occur at two stages: abnormal placentation early in the first trimester, followed by a "maternal syndrome" characterized by systemic vascular dysfunction in the later second and third trimesters. Various pathophysiologic mechanisms have been proposed for the placental dysfunction observed in stage 1, including OS. Specifically, inadequate spiral arteriolar remodeling by trophoblasts leads to narrow maternal vessels and relative placental ischemia. Intermittent hypoxia and reoxygenation caused by poor spiral artery invasion may result in OS, which promotes the transcription of antiangiogenic factors such as the soluble form of VEGFR-1 (sFLT1), leading to systemic vascular dysfunction that is also responsible for long-term cardiovascular impairment [74–76]. ROS can arise from various sources, such as mitochondrial stress or increased XO expression and activity caused by ischemia, as previously described [77]. The association between XO activity and HDP has been assessed in 9 of the 14 studies included in this systematic review.

The role of XO in PE was first investigated in a histological study by Many et al. in 2000 [21]. In this study, 10 placental specimens obtained from preeclamptic women were compared with 12 placental specimens from uncomplicated pregnancies. First, the immunohistochemical expression of XO was analyzed. In control samples, no XO expression in chorionic villi was found, whereas in PE samples, immunoreactivity was dramatically increased in the trophoblast layers, the stromal cores of blood vessels, and the invasive cytotrophoblasts. Furthermore, XDH/XO holoenzyme activity was compared with XO isoenzyme activity in placental villi and placental bed curettings from both cases and controls using a radiochemical method, which is one of the most sensitive methods for assessing XO activity. No difference in XDH/XO activity and XO activity was detected in placental villi samples between the PE and control groups. However, XDH/XO activity and XO activity in placental bed curettings (which contain invasive cytotrophoblasts) were significantly higher in the PE group compared with the control group. The invasive cytotrophoblast, with oxygenation determined by non-anastomosing basal arteries and diffusion through several cell layers of decidua, is more susceptible to hypoxia and, consequently, to increased XO activity, whereas the villous cytotrophoblast extensively exposed to intervillous blood flow is less subject to hypoxic stimulus. Despite similar XO activity in villous trophoblasts between groups, an increase in staining for nitrotyrosine was found in these cells, which can be considered an indirect marker of OS and endothelial dysfunction. Indeed, NO interacts with increased exposure to superoxide to form peroxynitrite, which causes nitration of tyrosine residues on proteins, forming nitrotyrosine. The resulting depletion of NO was assessed in the study included in the review conducted by V. Bambrana et al. [29], showing a decrease in NO in PE compared to healthy pregnant women before delivery. Therefore, the production of ROS, which interferes with the activity of NO synthase and the availability of NO, contributes to endothelial damage and reduced endothelial NO-mediated vasodilation, creating an altered vasomotor tone, as documented in some clinical studies in women with PE [78]. Finally, peroxynitrite, which belongs to reactive nitrogen species (RNS), can react with molecules such as proteins, DNA, and RNA, altering their structure and function [1]. Another histological study conducted by Shannon

A. Bainbridge et al., 2009 [27] demonstrated that the increase in XO activity is not limited to certain trophoblast subpopulations but also affects the skin of PE women. The authors found intense XO immunoreactivity within the stratum granulosum layer of the epidermis of skin biopsies collected from PE patients. The same result was observed in biopsies from patients with inflammatory conditions in active states (systemic lupus erythematosus, dermatitis, lichen simplex, mixed connective tissue disease, and bullous pemphigoid) [27]. Inflammatory cytokines such as TNF-alpha, IL-1beta, and IFN-gamma induced an increase in XO activity by 2, 2.5, and 8 times, respectively. This study emphasizes the importance of inflammation in PE, providing another potential factor contributing to the OS-induced progression of PE. XO overexpression in PE may be due not only to H/R mechanisms but also to inflammatory conditions caused by immune dysregulation and the release of apoptotic trophoblast cell residues into circulation [78]. In fact, in vitro studies have shown that elastase released by activated neutrophils induces the conversion of XDH to XO in endothelial cells, contributing to systemic OS [79]. In this regard, Ramya Rajshekar et al., 2021 [34] conducted a case–control study evaluating XO and plasma elastase levels in 30 PE patients and 30 normotensive pregnant individuals. In addition to confirming that XO levels were significantly higher in PE patients compared to controls, the authors demonstrated that elastase was 4.5-fold higher in PE patients than in controls. The difference in mean elastase levels was not statistically significant. Moreover, XO levels were positively correlated with elastase levels, confirming the role of leukocyte-induced activation in increasing XO activity in women with PE. As PE is a systemic condition, Abdulkadir Yildirim et al., 2004 [23] measured serum XO activity in 25 women with PE and 15 healthy pregnancies to assess whether the increased XO activity at the placental level in PE was also associated with increased systemic levels. The study found that mean plasma XO activity was higher in the PE group than in the healthy pregnancy group, confirming the systemic rather than local action of XO and OS. Moreover, this study also found a correlation between the severity of PE and the level of XO serum activity: XO activity was significantly higher in severe PE compared to mild PE. These findings were later confirmed in the study by Oguz Elmas et al., 2016 [31], which found that XO activity in 20 women with PE was higher than in controls and had strong correlations with blood pressure (diastolic, systolic, and mean arterial pressure), suggesting that XO activity increases proportionally with the severity of the condition (assessed in terms of blood pressure values). Additionally, according to ROC analysis, the authors found that the predictive values of XO activity and UA levels were significantly higher than those of allantoin. The study by V. Bambrana et al., 2015 [29] also assessed the role of XO in PE. The authors measured XO activity using a spectrophotometric method both during the antenatal and postpartum periods in 50 normal pregnancies and 50 PE pregnancies to determine if measuring XO is an accurate predictive marker for PE. They found that plasma XO activity in the PE group was 5.26 times greater than in normotensive pregnant women before delivery. Plasma XO activity decreased significantly after delivery in both PE and control groups but remained significantly greater (2.1 times) in the PE group vs. healthy controls ($p < 0.001$). In 2005 Aysun Bay Karabulut et al., 2005 [24] investigated the hypothesis that OS extends beyond the maternal compartment to the fetus. The authors analyzed not only maternal serum XO activity but also fetal plasma XO activity and MDA levels (a marker of lipid peroxidation) in maternal and cord plasma, finding that these parameters were also elevated in fetuses of PE pregnancies. Similarly, the study by M. Bogavac et al., 2012 [28] examined fetal conditions in PE pregnancies by measuring XO levels and activity in amniotic fluid samples from 66 normal pregnancies and 23 pregnancies complicated by gestational hypertension. The authors found a statistically significant difference in XO concentration and activity between the two study groups. Finally, Ilona Németh et al., 2002 [22] compared a group of healthy women with a group of pregnant women with gestational hypertension without signs of renal impairment, finding increased XO activity in hypertensive subjects. However, it is worth noting that in this study, XO activity was not measured in serum or placental lysates but was estimated indirectly as a ratio of urinary metabolites of caffeine after caffeine administration, as XO is involved

in the metabolism of methylxanthines. Two included studies (Abdulkadir Yildirim et al., 2004 [23] and Oguz Elmas et al., 2016 [31]) also analyzed XO activity alongside uric acid levels, which were found to be increased in PE women compared to controls. Some authors argue that the hyperuricemia observed in PE women is merely a secondary epiphenomenon due to reduced UA excretion caused by renal damage and increased tubular reabsorption due to hypovolemia, both characteristic of PE [80]. However, it could also result from increased XO activity, as it is the terminal product of purine metabolism. This hypothesis is corroborated by the evidence that serum uric acid to serum creatinine ratio, which normalizes uric acid for kidney function allowing for an indirect selection of patients with hyperactivation of XO, is elevated in women with PE and associated with adverse pregnancy outcomes [81]. Furthermore, hyperuricemia in PE patients may also play a pathogenetic role, as demonstrated by some in vitro studies showing that uric acid reduces trophoblast invasion into maternal uterine vessels, and murine models of hyperuricemia develop a PE-like syndrome during pregnancy. Finally, several observational studies found a correlation between hyperuricemia and the development of HDP [82–84]. Four included case–control studies (Abdulkadir Yildirim, 2004 [23]; Bogavac et al., 2012 [28]; Min Shang et al., 2015 [30]; Ebru Biberoglu et al., 2016 [32]) evaluated not only XO activity but also antioxidant protein levels in serum. Specifically, serum levels of SOD were found to be decreased in PE women compared to controls, likely due to consumption by excess of ROS. Also, at the placental level, immunohistochemical expression of SOD is reduced, as demonstrated by the histological study conducted by Many et al., 2000 [21].

4.5.2. XO and Gestational Diabetes

GD is the most common pregnancy complication, characterized by the onset of glucose intolerance during pregnancy. It affects about 10–15% of pregnancies and can lead to fetal macrosomia, prenatal mortality, and an increased long-term risk of developing type 2 diabetes mellitus [85,86]. Low-grade chronic inflammation and OS play central roles in the pathophysiology of GD. Risk factors for GD, such as obesity, are associated with increased numbers of resident adipose tissue macrophages that secrete pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β. This inflammatory state impairs insulin signaling and inhibits insulin release from β-cells [65]. Moreover, hyperglycemia induces OS through several metabolic mechanisms. Increased XO activity is also a key factor in this process [66]. Excessive ROS production inhibits insulin-stimulated glucose uptake by interfering with GLUT4 expression, contributing to the onset of GD [87]. These two pathophysiological mechanisms—low-grade inflammation and OS—are interconnected. Inflammatory cytokines stimulate XO activity and the conversion of XDH to XO, leading to increased superoxide radical production [56]. The association between GD and OS has also been demonstrated in animal studies, confirming the significant role of ROS in the onset of GD. For example, in a study using streptozotocin-induced diabetic pregnant rats, there was a depletion of the antioxidant defense system with decreased SOD and GSH activity and increased uric acid levels [88]. Despite the streptozotocin animal model (also referred to as a chemical model) being able to induce streptozotocin-induced fetal malformations [89], several authors have demonstrated that fetal malformations are due to OS and are clearly dependent on the embryonic levels/activity of antioxidant enzymes in genetic models of diabetes [66]. Of key importance is the immaturity of the fetal antioxidant system, making it particularly susceptible to the damaging effects of OS [66]. Indeed, there is evidence that maternal diabetes during pregnancy can induce OS in the fetus. For instance, a study by A. Biri et al., 2006 [25] investigated OS markers in the placenta, maternal plasma, and cord plasma in 13 pregnant women with GD and 13 women with normal glucose tolerance. They found significantly increased XO activity in maternal plasma and placental tissues in the GD group compared to controls, confirming the key role of XO in the pathophysiology of this disease. Higher levels of active XO were also detected in umbilical cord blood from fetuses of GD pregnancies, suggesting fetal ROS formation in this condition. The study by M. Bogavac et al., 2012 [28] also demonstrated increased fetal XO activity in amniotic

fluid samples from 18 women with GD and 66 women with uncomplicated pregnancies. Min Shang et al. assessed XO activity in maternal plasma, cord plasma, and placenta samples in two studies, one published in 2015 [30] and another in 2018 [33]. The 2015 study compared oxidative and antioxidant status in GD women diagnosed by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (requiring only a glucose value higher than the defined cutoffs during a 75 g 2 h oral glucose tolerance test) versus American Diabetes Association (ADA) criteria (requiring two or more glucose values above the cutoffs). They found OS in both subgroups, but women diagnosed by ADA criteria had higher maternal XO activity and, consequently, greater OS compared to those diagnosed by IADPSG criteria. This suggests that ADA criteria may identify more severe cases with poorer glycemic control. The study also found significant positive correlations between cord and placental XO levels and HbA1c values, further corroborating the hypothesis that women with poor glycemic control had higher levels of XO-induced OS. In the 2018 study, the authors evaluated the association between XO activity and the severity of GD by assessing markers of insulin resistance and sensitivity (HOMA-IR and QUICKI index, respectively). They found that women with GD had higher HOMA-IR and lower QUICKI compared to controls, indicating more severe insulin resistance. OS markers in maternal plasma, cord plasma, and placenta were positively correlated with HOMA-IR and negatively correlated with QUICKI, demonstrating that OS correlates with the severity of the condition. Additionally, levels of adipokines involved in insulin resistance were also positively correlated with XO activity, suggesting a role for OS in the pathogenesis of insulin resistance. Both studies by Min Shang et al. found that XO activity in maternal and cord plasma was negatively correlated with newborn birthweight, and macrosomic fetuses had increased XO levels in cord plasma, suggesting the neonatal impact of increased maternal XO and OS during pregnancy. Including XO measurements in the management of women with GD could not only improve the predictive and prognostic accuracy in this condition but also provide insights into the future cardiovascular risk of both the mother and the offspring.

4.5.3. XO and Intrauterine Growth Restriction

IUGR is defined as an estimated fetal weight below the tenth percentile for gestational age, typically arising in the second trimester of pregnancy. While the term "small for gestational age" (SGA) may encompass constitutionally small but healthy fetuses, IUGR signifies a pathological intrauterine growth retardation [90]. The causes of IUGR can be classified as fetal (e.g., chromosomal abnormalities, infections), maternal (e.g., nutritional deficiencies), or placental factors. Notably, uteroplacental dysfunction, such as abnormalities in uteroplacental blood vessels, accounts for 80% of IUGR cases [90].

Adequate extravillous trophoblast invasion and rapid villous angiogenesis are crucial for proper placental development and subsequent fetal growth. Altered maternal arterial remodeling has been linked to the pathophysiology of obstetric syndromes like IUGR through placental malperfusion. A key feature of the placenta in IUGR cases is the reduced volume, surface area, and vascularization of intermediate and terminal villi [91]. Inadequate development of spiral arteries impairs nutrient transport to the fetus and increases resistance within the umbilical circulation, leading to H/R, which exacerbates OS and damages placental tissue [37]. Several studies have reported increased OS markers in maternal and umbilical cord plasma, and placental tissues in pregnancies complicated by IUGR, confirming that OS plays a role in the condition [90]. An interesting study by A. Karowicz-Bilińska [90] demonstrated that the administration of arginine (a precursor of NO) and acetylsalicylic acid (an inhibitor of thromboxane synthase) reduced OS markers in IUGR pregnancies. These substances decrease thromboxane, which is involved in the increased generation of ROS. However, this study did not assess fetal weight and did not use XO as a marker of OS. In turn, XO activity could play a crucial role in the OS that characterizes this condition. A. Biri et al., 2007 [26] conducted a case–control study involving 13 singleton pregnancies complicated by IUGR and 12 healthy singleton pregnancies. Authors

investigated OS in women with IUGR compared to those with normal fetuses and found a significant increase in XO activity in the IUGR group in maternal and umbilical cord plasma, and placental biopsies. Another study (Ebru Biberoglu et al. [32]) published in 2016 assessed the relationship between IUGR and OS by comparing XO activity simultaneously in the circulation and myometrium. However, this study did not confirm the hypothesis of increased XO in IUGR pregnancies. The researchers found that serum and myometrial XO activity were comparable between the IUGR and control groups. Nevertheless, the authors observed higher malondialdehyde (MDA) concentrations, another marker of OS, in the serum and lower concentrations in the myometrial samples of the IUGR group compared to the control group. This suggests a "spillover" of MDA from the uterus to the circulation protecting the fetus from ROS. The conflicting results regarding OS markers in these studies could be attributed to the multifactorial nature of fetal growth restriction, which involves maternal, fetal, and placental pathologies. Additionally, potential biases, such as the inclusion of pregnancies with fetal weights below the tenth percentile but without growth restriction, may have influenced the outcomes.

4.6. Strategies for the Inhibition of Xanthine Oxidase during Pregnancy

Despite the fact that XO inhibitor drugs have been widely used for many years in the treatment of gout, there are no conclusive data regarding their safety during pregnancy.

Since risk cannot be ruled out, both allopurinol and febuxostat are currently classified as category C drugs, according to the original five-tier letter system of the Food and Drug Administration (FDA). According to the new FDA classification, the "Pregnancy and Lactation Labeling Rule" (PLLR), a more comprehensive narrative labeling system, allopurinol, and febuxostat belong to the group of drugs without well-controlled studies conducted in humans and/or adverse fetal effects in animal studies [92].

Regarding febuxostat, no adverse developmental effects and no teratogenicity were observed in embryo–fetal development studies, with oral administration to pregnant rats and rabbits during organogenesis at doses that produced maternal exposures up to 40 and 51 times, respectively, the exposure at the maximum recommended human dose (MRHD) [93]. As for allopurinol, the use of this drug in animal studies has induced species-specific reproductive toxicity. In humans, congenital malformations have been reported in some cases of newborns exposed to allopurinol. However, according to a systematic review from 2018 and another from 2024, the association between allopurinol and teratogenicity appears to be weak and with uncertain causality, and the currently available data are insufficient to make a definitive judgment [94,95].

To overcome the potential risks associated with the treatment of pregnant women with XO inhibitors, the incorporation of some foods with direct antioxidant activity, such as polyphenols, may be of help in pregnancy complications associated with an increased level and/or activity of XO. Several plant-derived bioactive compounds (luteolin, quercetin, isorhamnetin, galangin, prosapogenin, hesperetin, and theaflavin-3,3′-digallate) and dietary cranberry juice, purple grape juice, and black tea may inhibit XO activity [96,97].

Additionally, recent evidence suggests a protective role of certain bacteria isolated from fermented foods, which can prevent hyperuricemia by inhibiting purine absorption and XO activity [98]. It may also be beneficial to reduce the intake of certain foods, such as orange juice and pink grapefruit juice, which directly stimulate XO activity [97], as well as high purine-rich foods (such as animal meats, fish, organs like liver and fish milt, and yeast) that provide a large amount of substrate to the XO enzyme [99,100].

Finally, it must be considered that some medications commonly prescribed for HDP also have the pleiotropic effect of reducing OS through XO inhibition and/or the inhibition of free radicals production. For example, it has been demonstrated that certain beta-blockers [101,102], calcium channel blockers [103,104], magnesium sulfate [105], and acetylsalicylic acid [106] exhibit antioxidant properties.

This review has some limitations: firstly, XO was evaluated in different tissues and measured using different analytical methods in the included studies. Secondly, the total

number of included studies was low, with a low to moderate sample size in each study. Finally, patients were not stratified by age and gestational period.

5. Conclusions

The primary objective of this systematic review was to evaluate the existing studies on the associations between XO levels, activity, and/or expression at the fetal, maternal, and placental level, and major pregnancy pathologies such as HDP, GD, IUGR, PTB, and RPL.

The rationale for this research stems from previous studies demonstrating that OS is a key component of the pathophysiology of most frequent obstetric diseases and XO is an enzymatic marker of OS. Pregnancy-related pathological conditions are linked to altered placentation, leading to H/R processes. This, in turn, triggers the reversible or irreversible conversion of the XDH isoform to the XO isoform, which becomes an important source of ROS (especially superoxide and hydrogen peroxide), resulting in cellular and tissue damage. Moreover, H/R promotes apoptosis of trophoblast cells, leading to the shedding of cellular fragments into circulation, further exacerbating inflammation. Inflammatory cytokines also increase XO activity and expression, creating a vicious cycle. Major pregnancy complications such as PE and GD are also associated with an increased risk of future cardiovascular disease; studies outside pregnancy have demonstrated that XO and its terminal product uric acid are independently associated with cardiovascular risk. We therefore believe that, unlike other OS markers, assessing XO levels and activity could provide valuable information not only for pregnancy outcomes but also for the future risk of developing cardiovascular diseases later in life. Furthermore, since XO inhibitors are already used to treat symptomatic hyperuricemia, these medications may demonstrate in the future a cardioprotective role also in women with a history of pregnancy complications. Further studies are needed to evaluate the short- and long-term outcomes of patients with elevated XO activity during pregnancy. To the best of our knowledge, there are no studies that have investigated the correlation between XO levels in pregnant patients with the development of cardiovascular disease later in life. Additionally, no longitudinal studies have been conducted to monitor XO levels throughout pregnancy and after delivery, to identify a potential predictive role in disease onset, beyond its pathophysiological role.

All the studies but one (on women with IUGR fetuses) included in the present systematic review have demonstrated increased XO levels, activity, and/or expression in women diagnosed with HDP, GD, and IUGR. Moreover, the increase in XO is often systemic, affecting not only the placenta but also the entire maternal vascular system, and extending to the fetus, increasing the risk of fetal pathologies. However, no study has evaluated a potential association between XO and RPL or PTB.

Further evidence on the role of XO in pregnancy outcomes would not only deepen our understanding of the pathophysiological mechanisms at the basis of pregnancy complications but also potentially open new pharmacological possibilities for the prevention or treatment of obstetric pathologies characterized by increased XO activity. This would also contribute to the prevention of fetal health issues, as the fetus is exposed to maternal ROS and XO.

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