BMC Pregnancy and Childbirth

RESEARCH

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Impact of Vitamin D deficiency on immunological and metabolic responses in women with recurrent pregnancy loss: focus on VDBP/HLA-G1/CTLA-4/ENTPD1/adenosine-fetal-maternal conflict crosstalk

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Abstract

Background and aim Recurrent pregnancy loss (RPL), also known as recurrent implantation failure (RIF), is a distressing condition affecting women characterized by two or more consecutive miscarriages or the inability to carry a pregnancy beyond 20 weeks. Immunological factors and genetic variations, particularly in Vit D Binding Protein (VDBP), have gained attention as potential contributors to RPL. This study aimed to provide insight into the immunological, genetic, and metabolic networks underlying RPL, placing a particular emphasis on the interactions between VDBP, HLA-G1, CTLA-4, ENTPD1, and adenosine-fetal-maternal conflict crosstalk.

Methods A retrospective study included 198 women with three or more consecutive spontaneous abortions. Exclusion criteria comprised uterine abnormalities, endocrine disorders, parental chromosomal abnormalities, infectious factors, autoimmune diseases, or connective tissue diseases. Immunological interplay was investigated in 162 female participants, divided into two groups based on their Vit D levels: normal Vit D-RPL and low Vit D-RPL. Various laboratory techniques were employed, including LC/MS/MS for Vit D measurement, ELISA for protein detection, flow cytometry for immune function analysis, and molecular docking for protein–ligand interaction assessment.

Results General characteristics between groups were significant regarding Vit D and glucose levels. Low Vit D levels were associated with decreased NK cell activity and downregulation of HLA-G1 and HLA-G5 proteins, while CTLA-4 revealed upregulation. VDBP was significantly downregulated in the low Vit D group. Our findings highlight the intricate relationship between Vit D status and adenosine metabolism by the downregulation of SGLT1, and NT5E, key components of adenosine metabolism, suggests that Vit D deficiency may disrupt the regulation of adenosine levels, leading to an impaired reproductive outcome. HNF1β, a negative regulator of VDBP, was upregulated, while HNF1α, a positive regulator, was downregulated in low Vit D women after RPL. Molecular docking analysis revealed crucial residues involved in the interaction between Vit D and HNF1β.

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Conclusion Collectively, these findings underscore the importance of Vit D in modulating immune function and molecular pathways relevant to pregnancy maintenance, highlighting the need for further research to elucidate the mechanisms and potential therapeutic interventions for improving pregnancy outcomes in individuals with Vit D deficiency and RPL.

Keywords Recurrent pregnancy loss, Genetic variation, Vitamin D-Binding protein, CTLA-4 Antigen, Fetal-maternal conflict

Introduction

Recurrent pregnancy loss (RPL), also known as recurrent implantation failure (RIF), is a distressing condition that affects many women [1]. It occurs when a woman experiences two or more consecutive miscarriages or fails to carry a pregnancy beyond 20 weeks [2]. RPL can be emotionally challenging and hurt a woman's health and well-being. RPL causes are not fully understood but are believed to result from genetic and immunological factors [3]. Some common factors that contribute to RPL include inherited characteristics, such as abnormalities in the chromosomes of the developing embryo or a genetic predisposition to miscarriages [4]. Certain medical conditions, such as autoimmune, thyroid, or clotting disorders, can increase RPL risk [5].

Furthermore, the presence of alloimmune disorders and autoimmune abnormalities, such as antiphospholipid-antibodies (APAs), antithyroid antibodies, and anti-nuclear antibodies (ANAs), as well as issues with the cellular immune system, such as decreased inhibitory T cell numbers and increased quantities of natural killer (NK) cells, has been observed in women with RPL [6]. However, several immunological factors have gained attention in recent years as possible contributors to RPL [7]. Among these factors, the interplay between the human leukocyte antigen (HLA)-G1, HLA-G2, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has emerged as a potential area of interest [8]. HLA-G proteins are a group of proteins that are expressed on the surface of cells and play a role in immune regulation. HLA-G proteins are involved in developing tolerance to the fetus during pregnancy. In RPL, there is evidence that HLA-G protein expression is reduced, which may lead to an immune response against the fetus and result in miscarriage.

One hypothesis suggests that the HLA-G family involves decidual stromal cells (DSCs), the cells lining the uterus during pregnancy. DSCs play a crucial role in supporting the growth and development of the fetus. HLA-G family expression on DSCs has been shown to modulate pro-inflammatory immune responses, potentially contributing to the normal implantation process [9]. Impaired HLA expression or function in DSCs has been linked to RPL [10]. Studies have shown that HLA-G family expression is reduced in RPL patients, suggesting a potential role in the dysregulation of immune responses in the uterine environment. Several studies have investigated the role of HLA in RPL [11, 12]. One hypothesis suggests that the HLA-G family may regulate maternal and fetal immune cell balance. By interacting with inhibitory receptors on maternal T cells, the HLA-G family may help to prevent immune-mediated rejection of the developing fetus [13].

In addition, CTLA-4 is a co-stimulatory molecule crucial in downregulating immune responses [14]. It interacts with CD80 and CD86 on the surface of antigen-presenting cells, inhibiting T-cell activation. CTLA-4 − / − mice exhibit enhanced immune responses and are prone to miscarriage, highlighting the importance of CTLA-4 in immune regulation during pregnancy [15]. The interplay between HLA and CTLA-4 provides essential insights into the mechanisms involved in RPL [16]. HLA-G family suppresses immune responses, while CTLA-4 is critical in downregulating immune activation. Dysregulation of any of these molecules may result in an imbalance between immune tolerance and responses, ultimately leading to pregnancy loss [17]. Understanding the immunological interplay between these factors may provide new avenues for developing targeted therapies for RPL patients [18].

In the same context, Vitamin D (Vit D) is crucial for maintaining optimal reproductive health in women [19]. The receptors of this substance are located in several parts of the body, including the endometrium, placenta, deciduous cells, ovarian granule cells, uterine tube epithelium, pituitary gland, and hypothalamus [20]. By interacting with the Vit D receptor (VDR), Vit D is essential for controlling many biological processes, such as immune response and hormone synthesis. Vit D suppresses the adaptive immune system and affects the innate and acquired immunological responses [21].

Several studies have revealed a correlation between Vit D insufficiency and RPL, which is likely influenced by the activity of CD4 + T helper cells (Th) in both Th1 and Th2 immune systems [22]. It suppresses the production of Th1 cytokines, such as IFN-γ and tumor necrosis factor-alpha (TNF-α), and enhances the activity of Th2 by decreasing IFN-γ and increasing IL-4 (Fig. 1) [23, 24].

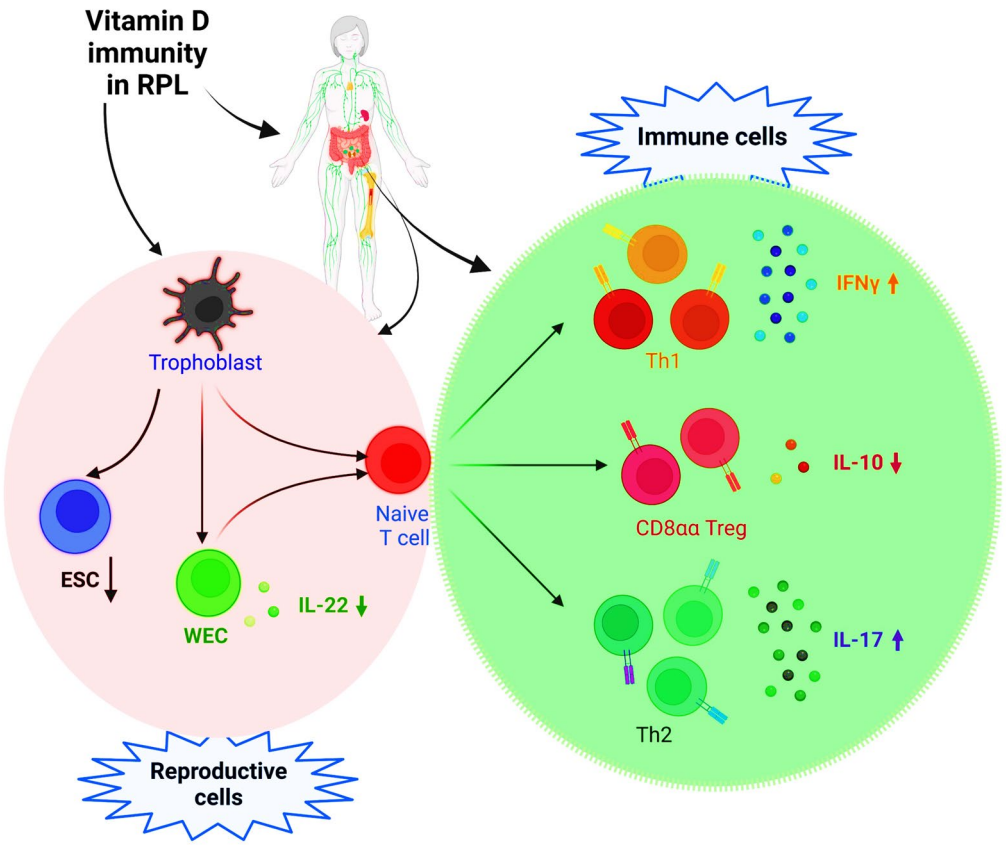

Fig. 1 A Vit D deficiency affects both the immune system and reproductive system. The effects of Vit D on immune cells such as B cells, T cells, Th cells (helper cells), macrophages (M-phages), and dendritic cells can be seen by understanding how Vit D affects maturation, differentiation, interleukin expression, and immunomodulatory functions. CD8aa Tregs highlights the diverse mechanisms through which Vit D modulates immune responses in RPL, including its potential impact on various Treg populations. Vit D modulates the expression of immunoreactive cytokines that are inflammatory in nature, which can affect the fetus' development. These cytokines are crucial in regulating the defense system and can affect the expression of genes and proteins and the differentiation and maturation of immune cells. Vit D can also act on trophoblast, cells that line the uterine wall and are responsible for forming the placenta. Abbreviations: ESC: endometrial stem cells; Th: T helper; WEC: whole endometrium cells; IL: interleukin; Treg: regulatory cells; CD: cluster of differentiation. RPL; recurrent pregnancy loss; Vit D: vitamin D; IFN-γ: Interferon-γ

Several women with RPL have a deficit in Vit D; consequently, these women have compromised cellular immune systems, characterized by increased amounts of peripheral NK cells, enhanced NK cell activity, and raised Th1/Th2 ratios [25]. Furthermore, women with low levels of Vit D are more prone to immunological abnormalities [26]. Further, Vit D binding protein (VDBP), also known as a group-specific component (GC), is a protein that transports Vit D in the body. It is crucial in regulating Vit D levels and maintaining calcium homeostasis [27]. In recent years, evidence has emerged suggesting a genetic interplay between VDBP and RPL [28].

One potential mechanism for VDBP involvement in RPL is its role in placental function. A dysfunctional placenta can lead to pregnancy complications due to the inability to exchange nutrients and oxygen [29]. It has been shown that VDBP is highly expressed in human placentas and that its deficiency impairs placental function [30]. Therefore, genetic variations in VDBP may alter its expression or function, potentially contributing to RPL. In addition, VDBP interacts with other proteins involved in reproductive processes. It interacts with steroid hormones such as estrogen and progesterone, essential for normal fetal development [31]. Due to genetic variations, VDBP's interactions

with these hormones may affect their bioavailability and signaling within the reproductive system. This, in turn, may contribute to some individuals' susceptibility to RPL [32]. Therefore, understanding the genetic pathway of Vit D in RPL can provide valuable insights into the underlying causes of this distressing condition. While further research is needed to fully elucidate the role of Vit D in RPL and unravel these genetic mechanisms, researchers could be able to identify novel therapeutic targets and improve the management of RPL [28].

In addition, metabolic interventions hold promise as pivotal strategies in optimizing pregnancy outcomes for individuals grappling with RPL [33]. By targeting adenosine signaling, purinergic pathways, and adenosine triphosphate (ATP) production, these interventions aim to address underlying metabolic dysregulations that may contribute to RPL [34]. Adenosine signaling plays a critical role in immune regulation and vascular function during pregnancy, and interventions aimed at modulating adenosine levels or activity could potentially mitigate inflammation and improve placental function [35]. Likewise, targeting purinergic pathways, including enzymes like ENTPD1 involved in adenosine metabolism, could restore balance to purinergic signaling and alleviate the burden of immune dysregulation in RPL [36, 37]. Additionally, enhancing ATP production may bolster cellular energy reserves crucial for embryonic development and implantation, strengthening the chances of a successful pregnancy [38]. These metabolic interventions represent promising avenues for personalized therapeutic approaches in RPL management, with the potential to improve pregnancy outcomes and alleviate the emotional burden associated with recurrent miscarriages [39].

Consequently, the causes of RPL are diverse, but a significant body of research has focused on the role of immunological factors and genetic variations in VDBP. This has led to the proposal of the theory that a lack of immunological factors and genetic variations in VDBP play a significant role in the occurrence of recurrent miscarriages. In light of this, this study sought to clarify the link between maternal blood levels of VDBP genetic variations and the immunological interplay of HLA-G1, HLA-G5, and CTLA-4 in RPL patients. Further, investigate the metabolic dysregulation observed in RPL, focusing on ENTPD1/NT5E and adenosine crosstalk. Additionally, *in-silico* studies were employed to identify genetic markers to predict RPL risk. This can then be used to develop screening tests to detect RPL early, allowing for more timely diagnosis and treatment.

Subjects and methods

The study was conducted retrospectively, including laboratory experiments and data collection. In this study, 198 women who had experienced three or more consecutive spontaneous abortions before 20 weeks of pregnancy were included with a history of recurrent pregnancy loss. These women were registered et al. Hadithah General Hospital, Al Qurayyat, Tabuk, Saudi Arabia, specializing in women's health from July 2023 to December 2023. The medical records of these women were reviewed in the order of their registration. Out of the total, 36 participants were not included in the study due to the following reasons: uterine abnormalities (7 cases); endocrine disorders, such as elevated prolactin levels, diabetes, and polycystic ovarian syndrome (21 cases); parental chromosomal abnormalities (2 cases); infectious factors (specifically *Chlamydia trachomatis*, 2 cases); and autoimmune diseases and/or connective tissue diseases (4 cases).

Practically, in the study, 162 female participants were divided into two groups to determine the immunological interplay between Vit D and RPL. Furthermore, a healthy women group ($n=70$) with a history of normal pregnancy and control participants had normal Vit D levels, which were confirmed through serum 25(OH)D measurements prior to their inclusion in the study. This was an essential criterion to establish a clear baseline for comparison with the RPL groups assigned to the previous groups to study relative mRNA fold changes and protein gene expression. This was done to compare the differences between the groups and to see if the mRNA fold change and protein expression of the healthy women group differed from those of the other groups. Illustratively, the first cohort had 89 women who experienced three or more consecutive pregnancy losses and had normal levels of Vit D (age range 22–39 years). The second cohort comprised 73 women who experienced three or more consecutive pregnancy losses and had low levels of Vit D (age range 21–41 years), as revealed in (Fig. 2). According to the Endocrine Society's clinical practice guidelines, Vit D deficiency is defined as a serum 25(OH) D level of less than 20 ng/mL, insufficiency as 21–29 ng/mL and sufficiency as 30 ng/mL or more [40]. For this study, low Vit D levels were defined as less than 20 ng/mL, which is consistent with these guidelines.

Women were enrolled, and blood samples were collected approximately 4 to 6 weeks after their last pregnancy loss. This time frame was maintained consistently across all three groups to reduce variability related to the immune system's response to the most recent pregnancy loss. For the control group, samples were collected at least 6 months postpartum, corresponding to the follicular phase of their menstrual cycle. This was done to

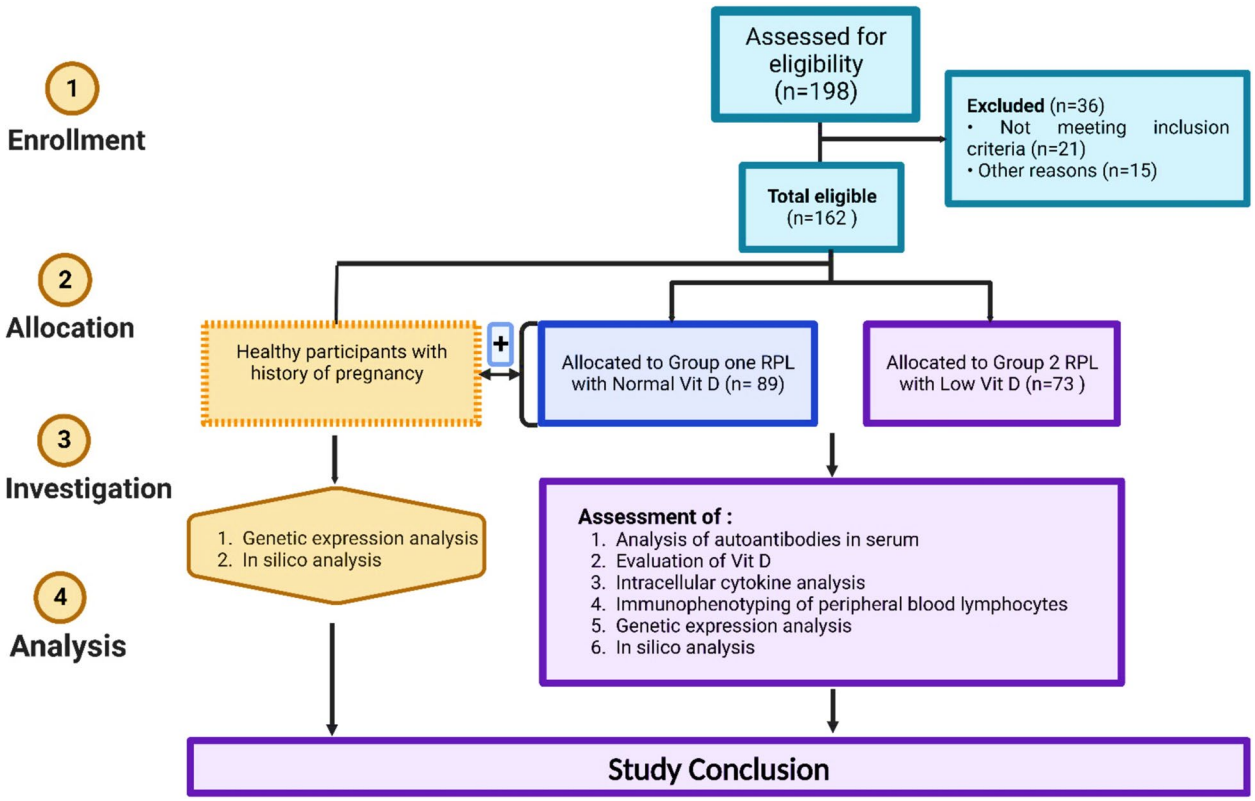

Fig. 2 An overview of the participation and enrollment process for participants. The enrollment process typically includes providing contact information, completing a registration form, and providing any necessary documentation

ensure that the hormonal and immunological profiles of the control group were comparable to those of the RPL groups at the time of sample collection.

A written informed consent was obtained from each participant before being involved in this study. The Ethical Committee for Medical Research of the University of Tabuk (UT-367–204-2024) granted permission for the planned research. The research was conducted in accordance with the Declaration of Helsinki. All participants were fully debriefed at the end of the study. All data was treated in strict confidence and kept confidential.

The eligibility criteria included women between 18 and 44 who were not pregnant. However, individuals who had undergone RPL examinations and women who could provide informed consent and were willing to participate in the study were considered eligible. Criteria for exclusion: Women who have previously had severe or uncontrolled medical conditions, such as cardiac, renal, hepatic, or endocrine diseases, which might potentially impact the results of their pregnancy; females with a history of prior therapy for Vit D deficiency or insufficiency; women who are using drugs that might potentially affect the metabolism of Vit D or the consequences of pregnancy; females who are currently pregnant or have intentions to conceive throughout the study; women who were previously dealing with drug misuse or mental conditions that might potentially hinder their ability to engage in the study thoroughly.

Women who were previously dealing with drug misuse or mental conditions that might potentially hinder their ability to engage in the study thoroughly. At the end of the procedure, 8 mL of blood was drawn and transferred into sterile EDTA and plain tubes. As soon as the blood coagulated, it was centrifuged at 1600×g for 5 min to extract serum for lab tests.

Analysis of Vit D, biochemical parameters, and autoantibodies

LC/MS/MS measuring Vit D levels using tandem mass spectrometry (Waters, USA) was performed [41]. ANA screening was conducted with indirect immunofluorescence using a kit from a commercial company (Elabscience Biotechnology Inc, Houston, USA). A commercially available kit (Merck KGaA, Darmstadt, Germany) was used to screen for autoantibodies against double-stranded DNA, single-stranded DNA, and histone. In a previous study by Blache et al. [42], intracellular cytokine analysis and peripheral blood immunophenotype were

conducted with flow cytometry (Beckman Coulter GmbH). ELISA was used to accurately measure the concentrations of ATP in the RPL samples using the ELISA kit (Catalog#:14432H1), purchased from MEIMIAN according to the manufacturer's protocols [34].

Intracellular cytokine analysis

Following the methodology reported in a previous study [43], flow cytometry was used to test the cytotoxicity of NK cells. A menstrual cycle follicular phase sample was used to assess NK cell cytotoxicity. The target cells were generated by cultivating the K562 cell line in RPMI 1640 medium containing 10% fetal bovine serum and 1% antibiotic-antimicrobial solution. A 37 °C humidified incubator with 5% CO_2 was used for cultivation. The K562 cells were washed twice in RPMI 1640 medium with an antibiotic–antimycotic solution and then suspended in 0.5 ml of PHK2 diluent from (Sigma Aldrich, St Louis, MO, USA).

Immunophenotyping of peripheral blood lymphocytes

Whole blood samples were labeled with fluorescently conjugated monoclonal antibodies (mAbs) targeting clusters of differentiations (CDs) for flow cytometry analysis. Labeling was performed using antibodies obtained from Beckman Coulter in Fullerton, CA, USA. The samples were processed using a Beckman Coulter FC 500 flow cytometer equipped with CXP software [44]. The manufacturer's instructions were followed to lyse the samples using Immuno-Lyse, and the samples were then washed twice using IsoFlow (Beckman Coulter GmbH).

Enzyme-linked immunosorbent assay (ELISA) and protein detection

Serum proteins were extracted using phosphate-buffered saline (PBS) at a concentration of 1X and pH 7.4. The protein concentrations were then measured using the Bradford protein assay (BioRad Labs, Milan, Italy). After standardizing the total protein concentrations of each sample to the lowest one, the levels of HLAs and CTLA-4 were quantitatively measured using competitive ELISA kits that were commercially available (Elabscience Biotechnology Inc, Houston, USA), following the instructions provided by the manufacturer. The samples were placed in 96-well plates previously coated with a capture antibody specifically targeting HLAs and CTLA-4, and the incubation period lasted 2 h. The wells were then rinsed thrice and incubated with a secondary antibody targeting HLAs and CTLA-4 coupled to horseradish peroxidase. Subsequently, the plates underwent three rounds of washing. A solution of H_2O_2 and tetramethylbenzidine was introduced as a substrate [45]. The optical density at 450 nm was then measured. The tests were performed three times to ensure accuracy and reliability.

cDNA synthesis, RNA extraction, and quantitative real-time PCR

Real-time PCR was conducted to measure VDBP, hepatocyte nuclear factor 1β (HNF1β), HNF1α, metalloproteinase-9 (MMP-9), MMP-2, and Vascular endothelial growth factor (VEGF), Glucose Transporter 1 (GLUT1), Glucose Transporter 3 (GLUT3), sodium-glucose cotransporter (SGLT-1), ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1), and 5'-nucleotidase ecto (NT5E) gene expression levels. We extracted mRNA from the samples and synthesized cDNA using the miRNeasy Mini Kit and QuantiTect Reverse Transcription Kit (Stemcell Technologies Inc, Catalog #07516). Using Enzynomics' qPCR Master Mix kit (Stemcell Technologies Inc, Catalog #07517), qRT-PCR was performed as the second step. For each qRT-PCR cycle, 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s were used [46, 47]. A list of the primers used is given in (Supplementary Table S1). In the comparative $2^{-\Delta\Delta Ct}$ method, endogenous β-actin was used as a housekeeping gene to calculate the relative expression of genes.

Molecular docking assessment

Instruments and tools

This study used the Windows 10 Pro operating system and Intel(R) Core (TM) i7-5500U CPU @ 2.40 GHz and DDR4 RAM. The Molecular Operating Environment (MOE 2022.02, Chemical Computing Group, Montreal, QC, Canada) software was used for protein and ligand retrieving and molecular docking.

Ligand preparation

The three-dimensional structure of 1,25-dihydroxy Vit D was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in SDF format and opened in MOE software for energy minimization and docking with target proteins.

Protein preparation

The three-dimensional structures of human's HNF1β, TNF-α, tumor necrosis factor receptor superfamily member 1*A* (TNFRSF1*A*), and tumor necrosis factor receptor superfamily member 1*B* (TNFRSF1*B*) were retrieved from the UniProt database (https://www.uniprot.org/). Target proteins were prepared for docking using MOE software by removing water and ligand molecules present in the protein structures along with target protein energy minimization.

Molecular docking analysis and visualization

Target proteins were docked with ligands using MOE software by identifying the binding site and docking with the induced fit model. Finally, the protein–ligand interactions were visualized using the same software.

Sample size calculation

A power analysis was accomplished to ascertain the clinical significance of the disparities between the groups. The expected sample size for low Vit D participants was 73 to get a power of 90% and a confidence interval of 95%. The power analysis calculated the minimal sample size required to detect a significant difference between the groups, considering the test's statistical power and the desired confidence level. Given a sample size of 73 participants, the probability of finding a statistically significant difference is 90%, assuming a 95% confidence range [48].

Statistical analysis

Statistical analyses were performed using GraphPad version 9.5.3. For non-parametric data, one-way analyses of variance were conducted using Kruskal–Wallis tests followed by Bonferroni corrections. Categorical frequency data were analyzed using chi-square or Fisher's exact test. One-way ANOVA followed by post-hoc Tukey and/or Games-Howell tests for multiple comparisons was employed to determine significant differences between the groups. All data were summarized as mean ± standard deviation (SD) and/or standard error of the mean (SEM) and median (interquartile range (IQR)). A *P*-value of 0.05 was used as the threshold for significance for all analyses.

Results

Demographic and clinical characteristics

The data from Table 1 highlight the demographic and clinical characteristics of women with three or more RPL, segmented by normal and low Vit D levels, compared to a control group. There are no significant differences in age, BMI, gravidity, and parity among the three groups, suggesting that these factors are not substantial distinguishing variables. The prenatal vitamin containing Vit D does not significantly differ between the normal and low Vit D groups ($p = 0.42$). The fasting blood sugar (FBS) levels are substantially higher in the normal Vit D group compared to the controls ($p = 0.045$) and markedly lower in the low Vit D group ($p = 0.041$), indicating a potential influence of Vit D on glucose metabolism. The median Vit D levels are similar between the control group (33 ng/mL) and the normal Vit D group (35 ng/mL), with a *P*-value of 0.128, indicating no significant difference. The control group has significantly higher median Vit D levels (33 ng/mL) compared to the low Vit D group (15 ng/mL), with a *P*-value of < 0.0001, indicating a highly significant difference. The normal Vit D group has significantly higher median Vit D levels (35 ng/mL) compared to the low Vit D group (15 ng/mL), with a *P*-value of < 0.0001, indicating a highly significant difference. This suggests that adequate Vit D levels might be associated with a better immune profile and potentially a lower risk of RPL. The consistent findings across the control and normal Vit D-RPL groups indicate that other factors, apart from Vit D, might influence the RPL risk.

Table 1 A comparison of the characteristics of women with healthy controls, normal vitamin D levels, and low vitamin D levels with three or more RPL

Characteristics	Control (*N* = 70)	Normal Vit D (*N* = 89)	Low Vit D (*N* = 73)	Control *vs* Normal Vit D (*P*-Value)	Control *vs* Low Vit D (*P*-Value)	Normal *vs* Low Vit D (*P*-Value)
Age (Years) (mean ± SD) Range	28 ± 2.89 (21–34)	32 ± 2.45 (22–39)	30 ± 1.59 (21–41)	0.566	0.325	0.125
BMI (Kg/m^2) (mean ± SD)	24.87 ± 1.89	25.32 ± 1.25	27.29 ± 2.04	0.611	0.523	0.22
Gravidity (Median (IQR))	2 (1–3)	2 (1–3)	2 (1–3)	0.965	0.897	0.58
Parity (Median (IQR))	1 (1–2)	0 (0–1)	0 (0–1)	0.745	0.056	0.78
IVF failure number (Median (IQR))	NA	0 (0–1)	0 (0–1)	NA	NA	0.92
Prenatal vitamin usage[a] (n%)	NA	36 (40.4%)	26 (35.6%)	NA	NA	0.42
FBS (mg/dL) (mean ± SD)	88.34 ± 2.56	102.23 ± 1.35	72.58 ± 2.11	0.045	0.041	0.021
Vit D levels (ng/ml) (Median (IQR))	33 (31–34)	35 (28–45)	15 (10–20)	0.128	< 0.0001	< 0.0001

Values were expressed as mean ± SD, or median, and IQR.

[a] Prenatal vitamin containing vitamin D 400 IU per day

NA: non-applicable. Significance at a *P*-value ≤ 0.05

Parameters of immune function and autoantibodies

The data in Table 2 reveals distinct differences in lymphocyte subsets between controls and women with RPL, particularly concerning Vit D levels. In women with low Vit D, there is a significant increase in CD3 + (T cells) compared to controls, suggesting a potential role for Vit D in modulating T cell responses. Both normal and low Vit D RPL groups showed elevated CD19 + (B cell) percentages compared to controls, with a further increase observed in the low Vit D group. This indicates a potential link between Vit D deficiency and B cell activation in RPL. Strikingly, CD56 + (NK cell) percentages are significantly different across all three groups, with the highest percentage observed in women with low Vit D RPL. This suggests a complex interplay between Vit D levels and NK cell activity in the context of RPL. Similarly, women with RPLs and deficiency in Vit D revealed a significantly higher NK cytotoxicity (%) compared with women with normal Vit D levels (Fig. 3a). As revealed in (Fig. 3b), an association between Vit D levels and NK cytotoxicity (%) was observed. A moderately negative correlation ($r = -0.35$, $P < 0.003$) was observed between NK cytotoxicity (%) and Vit D levels in all low Vit D-RPL. These results suggested that Vit D may regulate NK cytotoxicity in women with RPL and low Vit D levels with higher NK cytotoxicity.

The presence of various autoantibodies, including ANA, dsDNA, ssDNA, anti-histone, and anti-cardiolipin (IgG and IgM), is significantly higher in the low Vit D group compared to controls and the normal Vit D group, highlighting a potential link between Vit D deficiency and autoimmune activity. Notably, the Th1/Th2 cytokine ratio, as represented by TNF-α/IL-10 and IFN-γ/IL-10, shows significant variation, with the low Vit D group having a higher pro-inflammatory profile, which might contribute to adverse pregnancy outcomes in women with RPL (Table 2). These findings collectively suggest that low Vit D levels are associated with immune dysregulation and a higher prevalence of autoantibodies, potentially exacerbating the risk of RPL.

Assessment of immunological marker proteins and their correlation with Vit D levels in the studied groups

There was a significant downregulation in HLA-G1 and HLA-G5 protein and mRNA levels in RPL patients with low Vit D vs. RPL patients with normal Vit D samples (Fig. 4A-D). Interestingly, the levels of both HLA-G1 and HLA-G5 were the lowest in the control group and significantly lower than in the normal Vit D-RPL group despite similar Vit D levels. This suggests that while Vit D is crucial in regulating HLA-G1 and HLA-G5 expression, other factors inherent to the control group's immune response or regulatory mechanisms might influence these levels. On the contrary, upregulation of CTLA-4 protein was observed in the low Vit D group compared with the normal Vit D group (Fig. 4E-F). This suggests that Vit D may affect the expression of HLA-G1 and HLA-G5 proteins on one side and CTLA-4 on the other. Notably, gene expression by RT-PCR and protein analysis by ELISA revealed no significant differences between the two methods in the cases of gene expression and protein levels. While (Fig. 4) demonstrates significant differences in the expression of HLA-G1, HLA-G5, and CTLA-4 between women with normal and low Vit D

Table 2 Analyses of immune parameters and autoantibodies in women with healthy controls, normal and low vitamin D levels after three or more RPL

Characteristics	Controls ($N = 70$)	Normal Vit D-RPL ($N = 89$)	Low Vit D-RPL ($N = 73$)	Control vs Normal Vit D (P-Value)	Control vs Low Vit D (P-Value)	Normal vs Low Vit D (P-Value)
Lymphocytes						
CD3 (%)	70.00 ± 1.50	72.56 ± 1.89	77.25 ± 2.56	0.895	0.028	0.350
CD19 (%)	10.00 ± 0.50	14.23 ± 0.87	23.23 ± 0.45	0.041	0.002	0.020
CD56 (%)	7.00 ± 0.30	9.32 ± 0.74	24.45 ± 0.78	0.0001	0.0001	0.0001
Autoantibodies						
ANA	0/70 (0.0%)	11/89 (12.3%)	33/73 (45.2%)	0.001	0.0001	0.045
dsDNA	1/70 (1.4%)	4/89 (4.4%)	11/73 (15.1%)	0.001	0.001	0.030
ssDNA	1/70 (1.4%)	9/89 (10.1%)	14/73 (19.2%)	0.001	0.001	0.045
Anti-histone	0/70 (0.0%)	3/89 (3.3%)	7/73 (9.5%)	0.039	0.003	0.060
Anti-cardiolipin IgG	0/70 (0.0%)	6/89 (6.7%)	9/73 (12.3%)	0.004	0.002	0.050
Anti-cardiolipin IgM	0/70 (0.0%)	5/89 (5.6%)	10/73 (13.6%)	0.003	0.003	0.040
Th1/Th2 ratio						
TNF-α/ IL-10	41.45 ± 2.00	42.23 ± 1.99	38.78 ± 2.35	0.213	0.048	0.370
IFN-γ/IL-10	9.80 ± 0.50	11.23 ± 0.89	14.23 ± 0.25	0.347	0.032	0.250

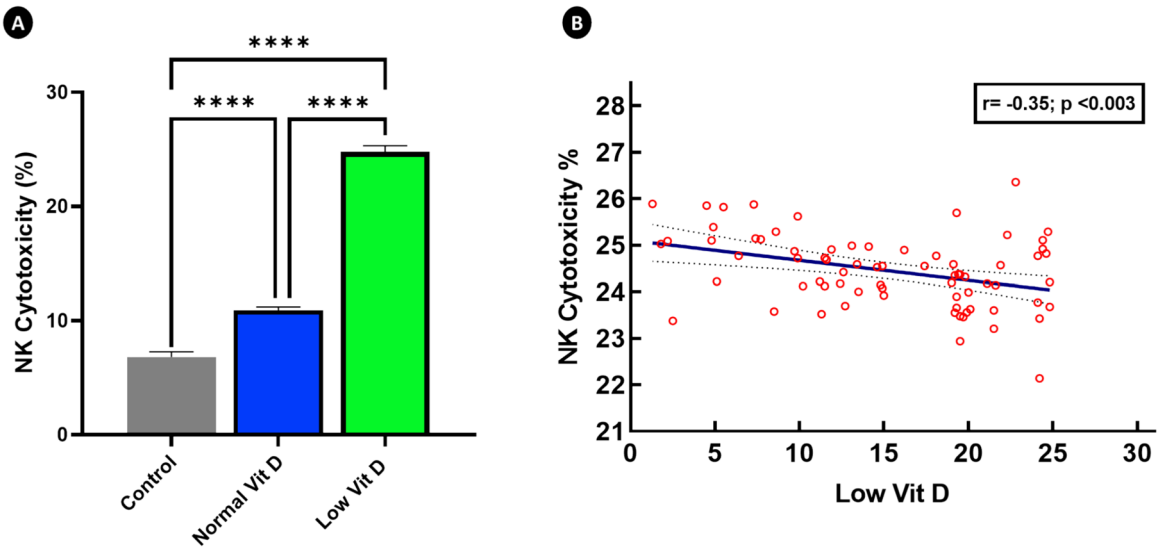

Fig. 3 **A** Comparison of NK cytotoxicity (%) in women with recurrent pregnancy losses and Vit D deficiency or normal levels. **B** A correlation between NK cytotoxicity (%) and Vit D levels in low Vit D-RPL patients. Women with recurrent pregnancy losses and low Vit D levels had significantly higher NK cytotoxicity (%), suggesting that Vit D deficiency may lead to those losses. The bar represents mean levels (± SEM). One-way ANOVA followed by post-*hoc* Games-Howell test was employed to determine the significant differences ($P < 0.05$). **** ($P \leq 0.0001$) compared to control

levels, Fig. 4g-i reveals varying strengths of correlation between Vit D levels and these markers across all participants. This suggests that while Vit D status influences the expression of these immune markers, other factors may also contribute to their regulation.

Spearman's correlation coefficient determined how Vit D was correlated with HLA-G1, HLA-G5, and CTLA-4 markers (Fig. 4g-i). It was observed that HLA-G5 and HLA-G1 exhibited strong and moderately significant negative correlations (r=-0.72, $P<0.0001$), (r=-0.67, $P<0.0001$), respectively. In contrast, CTLA-4 had a weak positive correlation with Vit D ($r=0.27$, $P=0.023$). Since Vit D is negatively correlated with HLA-G1 and HLA-G5 expression, it suggests that Vit D may play a role in suppressing the expression of these markers, whereas CTLA-4 appears to be positively affected by Vit D. It would therefore appear that Vit D may function to regulate the immune system through its effects on HLA-G1 and HLA-G5.

Expression levels of key genes

Real-time PCR was used for the determination of relative mRNA levels for the VDBP, HNF1β, and HNF1α transcription factors (Fig. 5a-c). It was found that VDBP exhibited a greater level of downregulation in the RPL women with low Vit D levels compared to healthy women with intact Vit D levels ($p<0.0001$) and RPLs females with normal levels of Vit D ($p=0.01$). The mRNA levels of the HNF1β transcription factor were significantly ($p<0.0001$) higher in low Vit D-RPL women compared to both healthy ($p<0.0001$) and normal Vit D-RPL women. Conversely, the HNF1α transcription factor displayed the lowest mRNA levels in low Vit D-RPL women in comparison to both healthy ($p<0.0001$) and normal Vit D ($p<0.0001$) women. Collectively, expression levels were significantly lower (VDBP and HNF1α) or higher (HNF1β) in women with RPL and normal Vit D compared to control. This suggests that HNF1β is more sensitive than HNF1α to changes in Vit D levels and may be a better indicator of Vit D deficiency. Except for MMP2 in low Vit D-RPL versus the normal Vit D-RPL, real-time PCR data of MMP-9 and VEGF genes involved in the angiogenesis revealed a significant downregulation of all genes among RPL patients with low levels of Vit D when compared to the control healthy and normal Vit D-RPL women (Fig. 5d-f). This data suggest that Vit D may regulate angiogenesis and proliferation genes through its regulation of expression and activity and that low levels of Vit D may be associated with an increased risk of RPL.

Vit D, adenosine, and glucose transporters: Intertwined metabolic dysregulation pathways and crosstalk in low Vit D-RPL

The investigation into the relationship between Vit D levels among women experiencing RPL and altered glucose metabolism via GLUT1, GLUT3, and SGLT-1 expression has revealed intriguing findings. Specifically, the study observed significantly elevated GLUT1, GLUT3, and SGLT-1 expression levels in women with normal Vit D-RPL levels compared to those with low Vit D-RPL

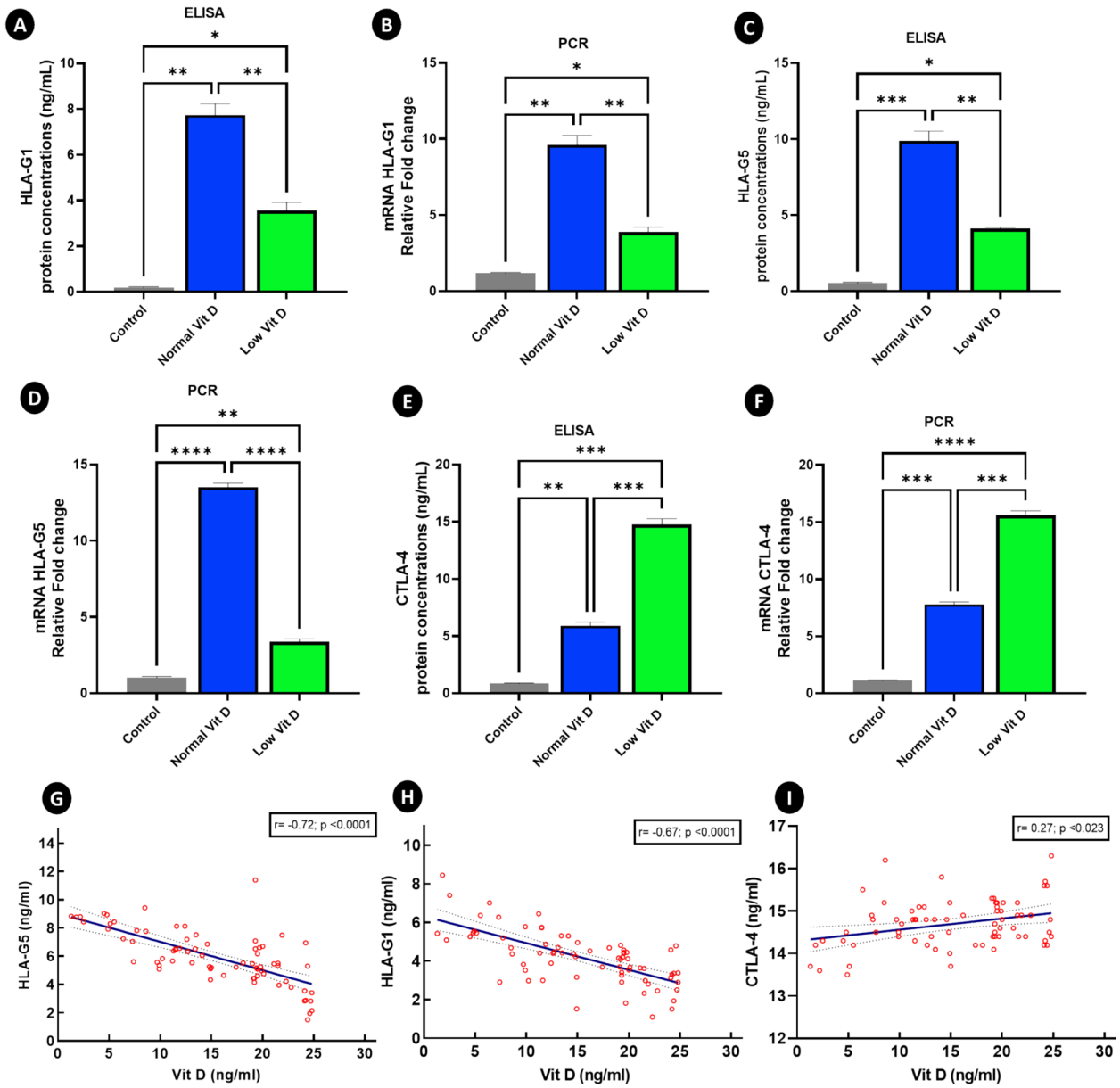

Fig. 4 **A** Protein concentration of HLA-G1; **B** Relative Fold change of HLA-G1; **C** Protein concentration of HLA-G5; **D** Relative Fold change of HLA-G5; **E** Protein concentration of CTLA-4; **F** Protein expression level of CTLA-4. Spearman correlation analysis between protein concentrations (ELISA values) for **G** HLA-G5; **H** HLA-G1; **I** CTLA-4 and Vit D in low Vit D-RPL patients. The bar represents mean levels (± SEM). One-way ANOVA followed by post-*hoc* Tukey's multiple comparisons test was employed to determine the significant differences ($P < 0.05$). * ($P \leq 0.05$), ** ($P \leq 0.01$) *** ($P \leq 0.0001$) compared to control. The value of 'r' denotes the Spearman's correlation coefficient; (−) = Negative correlation

levels and healthy controls (Fig. 6a-c). This may be attributed to compensatory mechanisms that maintain glucose homeostasis despite Vit D deficiency. Additionally, the slight decrease in SGLT1 expression in the low Vit D-RPL group may reflect a specific effect of Vit D deficiency on this transporter.

The investigation into ATP concentrations across three groups categorized by Vit D status has revealed notable variations. Among the Control group, ATP concentrations ranged from 5.26–7.89 ng/mL, indicating a moderate level of ATP production. In contrast, the normal Vit D-RPL group exhibited higher ATP concentrations, ranging from 7.899 ng/mL to 9.325 ng/mL, suggesting elevated ATP production associated with normal Vit D-RPL levels. Conversely, the Low Vit D-RPL group displayed notably lower ATP concentrations, ranging from

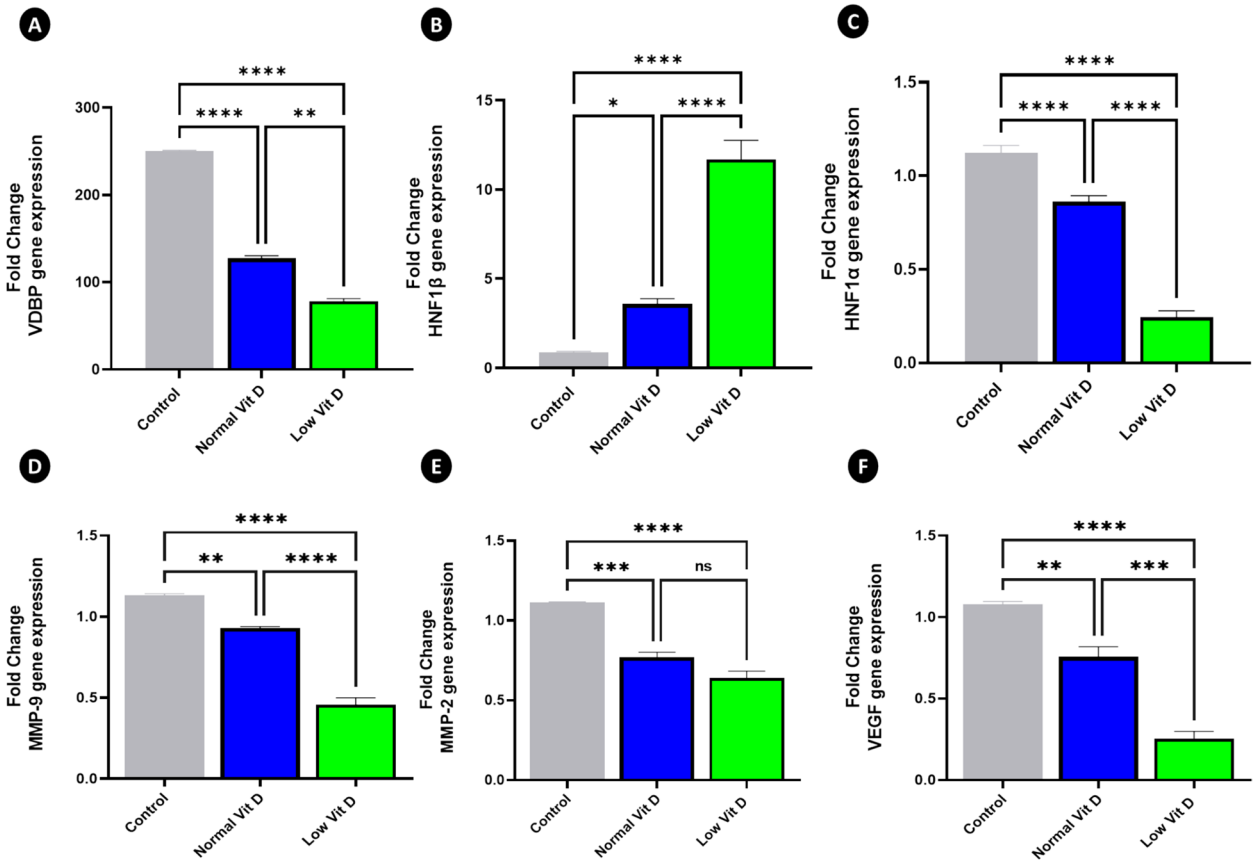

Fig. 5 RT-PCR analysis of fold expression of the target genes. **A** VDBP was more downregulated in low Vit D patients. **B** HNF1β transcription factor (negative regulator for VDBP). **C** HNF1α transcription factor (positive regulator for VDBP). **D** MMP-9 was more downregulated in low Vit D patients. **E** MMP-2. **F** VEGF. The graphs of genes are plotted as $2^{-\Delta\Delta Ct}$ fold changes. Data were expressed as mean ± SEM. One-way ANOVA followed by post-*hoc* Tukey's multiple comparisons test was employed to determine the significant differences ($P < 0.05$). * ($P \leq 0.05$), ** ($P \leq 0.01$) **** ($P \leq 0.0001$) compared to control

1.899–3.11 ng/mL, indicating reduced ATP production in the context of Vit D-RPL deficiency (Fig. 6d). These findings suggest a potential correlation between the low Vit D-RPL group and metabolic dysregulation of adenosine.

Specifically, the study observed significantly elevated expression levels of both ENTPD1/NT5E in women with normal Vit D-RPL levels compared to those with low Vit D-RPL levels. Conversely, women with low Vit D-RPL levels displayed notably lower expression levels of ENTPD1/NT5E. This observed pattern suggests a potential correlation between Vit D-RPL status and ENTPD1/NT5E expression levels (Fig. 6e-f). These findings underscore the possible role of Vit D in modulating adenosine pathway metabolism and its crosstalk with ENTPD1/NT5E in the context of recurrent pregnancy loss.

Molecular docking analysis

Data represented in (Fig. 7a-c) revealed the molecular interaction of 1,25-dihydroxy Vit D with ASN133 (H-donor), LYS237 (H-acceptor), and ARG137 (H-acceptor) residues in the binding site of HNF1β with energy of -6.67 kcal/mol. This indicates that these residues are essential for the binding between 1,25-dihydroxy Vit D and HNF1β and that the binding energy is strong and likely to be stable.

By H-donor, 1,25-dihydroxy Vit D bound with ASP140 residue in the binding site of TNF-α with -6.07 kcal/mol of energy (Fig. 7d-f). This suggests that ASP140 is a crucial residue for binding 1,25-dihydroxy Vit D to the binding site of TNF-α.

A similar interaction was observed with the binding site of TNFRSF1*A* with energy of -6.75 kcal/mol (Fig. 7g-i), whereas it responded to the binding site of TNFRSF1*B* with a -5.84 kcal/mol (Fig. 7j-l). This indicates that the binding energy between the TNFRSF1*A* and TNFRSF1*B* is slightly different, with the TNFRSF1*A* having somewhat more vital binding energy. This could be due to the slightly different structure of the TNFRSF1*A* and TNFRSF1*B* proteins.

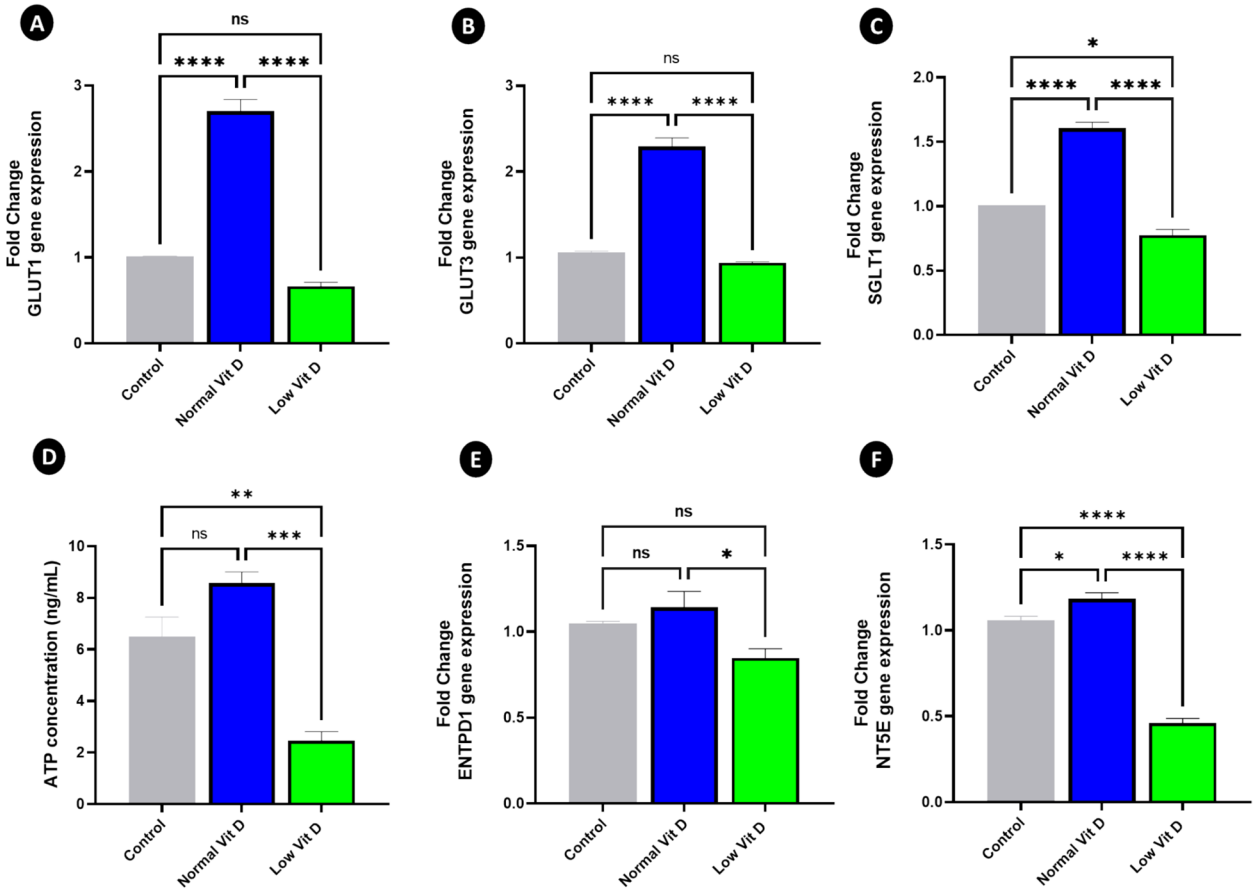

Fig. 6 Impact of Vit D Deficiency on Glucose and Adenosine Metabolism in RPL. **A** GLUT1, **B** GLUT3, **C** SGLT1. **D** ATP levels measured by ELISA, and gene expression as measured by RT-PCR for target genes **E** ENTPD1 and **F** NT5E. Data were expressed as mean ± SEM. The graphs of genes are plotted as $2^{-\Delta\Delta Ct}$ fold changes. The control group consists of healthy women with normal Vit D levels. Data were expressed as mean ± SEM. One-way ANOVA followed by post-*hoc* Tukey's multiple comparisons test was employed to determine the significant differences. ^{ns}: non-significant ($P < 0.05$). * ($P \leq 0.05$), ** ($P \leq 0.01$) **** ($P \leq 0.0001$) compared to control

Discussion

The cause of RPL is unknown in about 50% of cases, but it affects close to 1% of couples [4]. In some instances, RPL may be overlooked because of autoimmune dysregulation, which has been revealed to be a probable cause [49].

Vit D is an essential nutrient that plays a crucial role in various bodily functions. A growing body of research has recently suggested a link between Vit D deficiency and autoimmune diseases [50]. Autoimmune diseases are characterized by the inappropriate immune system attack on healthy tissues, leading to inflammation and tissue damage. One area of concern is the relationship between Vit D deficiency and RPL [51].

Several studies have shown that women with Vit D deficiency are at an increased risk of experiencing RPL. For instance, one study involving 100 women with RPL found that 82% had Vit D levels below the recommended range [52]. Moreover, studies in animal models have suggested that deficiency of Vit D can lead to

(See figure on next page.)

Fig. 7 The Molecular Interactions between Vit D and HNF1*β*, TNF-*α*, TNFRSF1*A* and TNFRSF1*B*. **A** 2D binding interactions between Vit D and HNF1*β*; **B** 3D active pocket binding mapping between Vit D and HNF1*β*; **C** Surface binding mapping between Vit D and HNF1*β*. **D** 2D binding interactions between Vit D and TNF-*α* **E** 3D active pocket binding mapping between Vit D and TNF-*α*; **F** Surface binding mapping between Vit D and TNF-*α*. **G** 2D binding interactions between Vit D and TNFRSF1*A*; **H** 3D active pocket binding mapping between Vit D and TNFRSF1*A*; **I** Surface binding mapping between Vit D and TNFRSF1*A*. **J** 2D binding interactions between Vit D and TNFRSF1*B*; **K** 3D active pocket binding mapping between Vit D and TNFRSF1*B*; **L** Surface binding mapping between Vit D and TNFRSF1*B*

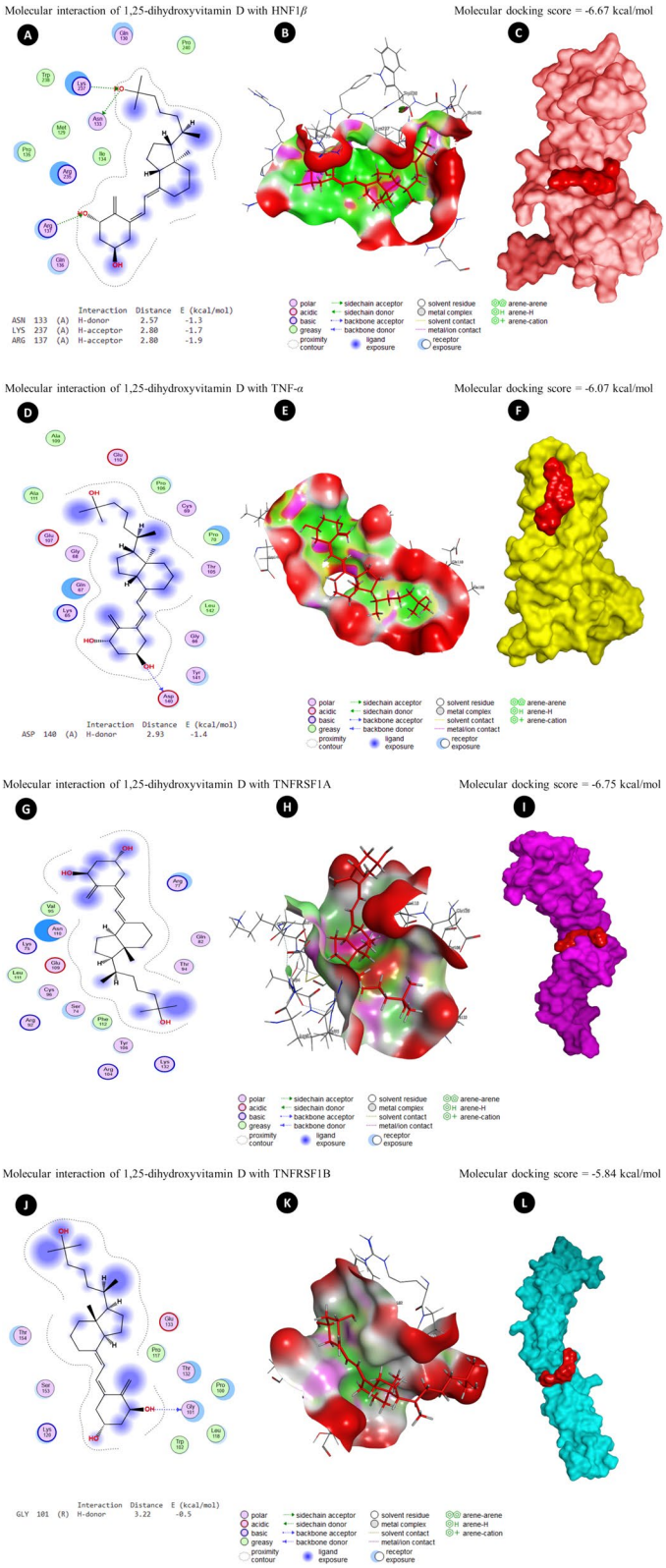

Fig. 7 (See legend on previous page.)

autoimmune diseases, such as systemic lupus erythematosus (SLE), the most common autoimmune condition affecting people with RPL [53, 54].

The exact mechanisms by which Vit D deficiency contributes to autoimmune disease and RPL are not fully understood. However, Vit D deficiency is thought to disrupt the balance between immune cells and regulatory T cells, resulting in an impaired immune response against self-antigens [26, 54]. This dysregulation of the immune system, in turn, can contribute to the development of autoimmune diseases and increase the risk of pregnancy complications, including RPL [55].

Given the importance of Vit D in maintaining immune function and reproductive health, women with RPL must consider having their Vit D levels checked by their healthcare provider. Supplementation with Vit D, if indicated, can help optimize Vit D levels and reduce the risk of autoimmune disorders and RPL [56]. In this study, women's general characteristics were non-significant; this is consistent with D'Ippolito et al., [57], who showed that the four groups in their sample did not differ significantly regarding distributions of general characteristics, such as age, employment status, menstruation cycle duration, body mass index. This could be due to the fact that the study was focused only on the differences in health outcomes, and any differences between the groups in terms of general characteristics were likely not significant enough to have a measurable effect on health outcomes.

Furthermore, our data revealed that Vit D deficiency is associated with increased activity of the NK cells compared to the normal Vit D-RPL and control groups. Vit D deficiency could increase the autoimmunity of those with certain diseases due to the increased activity of NK cells. Therefore, adequate Vit D levels are necessary for RPL patients. Several studies have revealed that Vit D can modulate the activity of immune cells, including NK cells [58, 59]. Vit D receptors (VDRs) have been identified in NK cells, suggesting that Vit D can directly interact with them. This interaction has been revealed to influence the activation and function of NK cells [60].

Our findings indicate a significant downregulation in HLA-G1 and HLA-G5 protein and mRNA levels in RPL patients with low Vit D vs. RPL patients with normal Vit D. However, There were lower levels of HLA-G1 and HLA-G5 in the control group compared to the normal Vit D-RPL group, despite similar Vit D levels, suggest that Vit D may not be the sole regulator of these proteins. Other factors associated with RPL, such as inflammation or oxidative stress, could influence the expression of HLA-G1 and HLA-G5. Additionally, the lower levels in the control group may reflect the physiological state of healthy pregnancies, where a balance of immune tolerance and activation is crucial for successful implantation and fetal development.".

Regarding the correlations, the moderate negative correlation between HLA-G1 and Vit D levels indicates a trend where lower Vit D levels are associated with higher HLA-G1 expression, although the relationship is not as strong as that observed for HLA-G5. The weak positive correlation between CTLA-4 and Vit D levels suggests a potential association, but further investigation is needed to clarify the nature of this relationship. The observed differences in correlation strengths between Vit D levels and HLA-G1, HLA-G5, and CTLA-4 expression could be attributed to several potential mechanisms: firstly, the expression levels of Vit D receptors (VDRs) may vary among different immune cell types. HLA-G1 and HLA-G5 are primarily expressed on extravillous trophoblasts and NK cells, while CTLA-4 is expressed on activated T cells. If VDR expression is higher in cells expressing HLA-G1 and HLA-G5 compared to CTLA-4-expressing cells, it could explain the stronger negative correlation observed for HLA-G1 and HLA-G5. Secondly, Vit D binding to VDRs initiates signaling cascades that can modulate gene expression. These signaling pathways may differ between immune cell types, leading to varying effects on HLA-G1, HLA-G5, and CTLA-4 expression. For example, Vit D may suppress HLA-G1 and HLA-G5 expression by inhibiting pro-inflammatory cytokines, while its impact on CTLA-4 may be mediated through different mechanisms. Thirdly, Vit D has been shown to influence epigenetic modifications, such as DNA methylation and histone acetylation, which can regulate gene expression. These epigenetic changes may differentially affect the expression of HLA-G1, HLA-G5, and CTLA-4, leading to varying responses to Vit D levels. Lastly, The expression of HLA-G1, HLA-G5, and CTLA-4 may be influenced by other factors, such as cytokines, hormones, and genetic variations. These factors may interact with Vit D signaling, leading to complex regulatory mechanisms that could explain the observed differences in correlation strengths.

Furthermore, this study explored the connection between serum Vit D and VDBP in women with RPL. We found that the VDBP was more downregulated in the low Vit D group than in the normal one. This suggested that VDBP plays an essential role in maintaining adequate levels of Vit D during pregnancy and that low levels of VDBP can lead to RPL [61]. Thus, our study provides evidence that levels of Vit D and VDBP are closely connected and that low levels of VDBP can contribute to RPL. Several studies have revealed that low levels of VDBP are associated with a higher risk of RPL [62, 63]. According to the study of Yan et al. [61], the women with RPL had significantly lower VDBP levels than those with

successful pregnancies. In another study by Ota et al., Vit D supplementation was tested in women with RPL [64]. According to this study, women receiving high doses of Vit D along with VDBP supplements had a significantly higher pregnancy rate than women receiving placebos. So, based on our findings, it may be possible that low levels of VDBP contribute to RPL development. Improved fertility outcomes and reduced RPL are possible by optimizing Vit D and VDBP levels [65].

The findings from our study, examining the relationship between Vit D levels, altered glucose metabolism via GLUT1, GLUT3, and SGLT-1 expression, and other metabolic markers in women experiencing RPL, shed light on the intricate metabolic adaptations occurring during pregnancy and their potential implications for pregnancy outcomes. Pregnancy is characterized by dynamic changes in glucose metabolism, with a gradual increase in insulin resistance serving as a physiological adaptation to ensure an adequate supply of glucose to the rapidly growing fetus [66, 67]. Our results indicate that women with normal Vit D-RPL levels exhibit significantly elevated expression levels of GLUT1, GLUT3, and SGLT-1, suggesting that Vit D levels alone may not be the sole determinant of glucose metabolism dysregulation in RPL. Other factors associated with RPL, such as inflammation or oxidative stress, could influence these glucose transporters' expression [68]. Conversely, women with low Vit D-RPL levels display slightly lower SGLT-1 expression levels, highlighting a potential dysregulation in glucose metabolism associated with Vit D deficiency [69]. Specifically, the study observed significantly elevated GLUT1, GLUT3, and SGLT-1 expression levels in women with normal Vit D-RPL levels compared to those with low Vit D-RPL levels and healthy controls. Similar transcript levels in the low Vit D-RPL group and healthy controls may also indicate a compensatory mechanism to maintain glucose homeostasis despite Vit D deficiency. These mechanisms could involve the upregulation of other glucose transporters or alternative pathways for glucose uptake. Additionally, the slight decrease in SGLT1 expression in the low Vit D-RPL group may reflect a specific effect of Vit D deficiency on this transporter, although the exact mechanism remains unclear.

The observed pattern of GLUT1, GLUT3, and SGLT-1 expression levels aligns with the physiological adaptations during pregnancy, where insulin resistance enhances glucose availability for fetal growth and development [70]. During early gestation, the embryo relies on glycolysis to produce ATP, with glycogen as the primary energy source [71]. However, our findings suggest that deficiencies in glycogen and glucose metabolism, indicated by low expression of GLUT1, GLUT3, and SGLT-1 genes, may lead to reduced ATP and adenosine levels, potentially impacting embryonic development and pregnancy maintenance [72].

In addition, adenosine, a key signaling molecule involved in various physiological processes, including immune regulation and vascular function, plays a crucial role in maintaining pregnancy [73]. Our findings indicate that women with low Vit D-RPL levels exhibit significantly lower expression levels of ENTPD1 and NT5E, enzymes involved in adenosine metabolism, compared to those with normal Vit D-RPL levels. The balance between ENTPD1-mediated ATP hydrolysis intricately regulates adenosine metabolism to adenosine monophosphate (AMP) and NT5E-mediated conversion of AMP to adenosine [74]. The observed downregulation of ENTPD1 and NT5E expression in low Vit D-RPL women suggests a potential dysregulation in adenosine metabolism, leading to altered adenosine levels and signaling [75]. This dysregulation may have significant implications for pregnancy outcomes, as adenosine plays critical roles in immune modulation, trophoblast invasion, and placental development. However, while we observed significantly higher ENTPD1/NT5E expression in the normal Vit D-RPL group compared to the low Vit D-RPL group, ATP levels were also elevated in the normal Vit D-RPL group (Fig. 6d). This observation contradicts the expected relationship between ENTPD1/NT5E and ATP, where higher enzyme expression would typically lead to lower ATP levels due to increased degradation. Several factors could explain this discrepancy. The increased ENTPD1/NT5E expression in the normal Vit D-RPL group could be a compensatory mechanism to maintain ATP levels by increasing the efficiency of ATP breakdown and recycling. Alternatively, other regulatory factors not examined in this study, such as increased ATP production or decreased ATP consumption, could be counteracting the effects of increased ENTPD1/NT5E expression. Additionally, the timing of sample collection may have influenced the results, as the expression of ENTPD1/NT5E and ATP levels may fluctuate throughout the menstrual cycle or during different stages of pregnancy. Further research is needed to elucidate the mechanisms underlying this observed relationship between ENTPD1/NT5E expression and ATP levels in women with RPL. Also, our findings highlight the intricate relationship between Vit D status and adenosine metabolism. The downregulation of GLUT1, GLUT3, and SGLT1, critical components of glucose transport in the low Vit D-RPL group, suggests that Vit D deficiency may disrupt glucose metabolism. However, this downregulation was not observed compared to the control group, indicating that other factors may also be involved. Impaired glucose metabolism could

reduce ATP production, as glucose is a cell's primary energy source. Additionally, the downregulation of ENTPD1/NT5E, enzymes involved in adenosine production and signaling, suggests that Vit D deficiency may disrupt the regulation of adenosine levels. These ATP and adenosine level alterations could contribute to an impaired reproductive outcome, as both molecules play crucial roles in various physiological processes during pregnancy.

Moreover, the crosstalk between ENTPD1/NT5E and adenosine pathways is complex and bidirectional [76]. Adenosine acts as a feedback inhibitor of ENTPD1 activity, while ENTPD1-derived adenosine regulates adenosine receptor signaling and downstream cellular responses [77]. Therefore, dysregulation of ENTPD1/NT5E expression may disrupt this delicate balance, leading to aberrant adenosine signaling and potential adverse effects on pregnancy [78]. The observed metabolic dysregulation in low Vit D-RPL women underscores the importance of Vit D in modulating adenosine pathway metabolism and its crosstalk with ENTPD1/NT5E. Vit D has been implicated in immune regulation and inflammation modulation, and its deficiency may exacerbate the dysregulation of adenosine metabolism, contributing to the pathogenesis of RPL. Therefore, any future intervention to restore Vit D levels and modulate adenosine metabolism may hold promise as potential therapeutic strategies for improving pregnancy outcomes in women with RPL [79].

Further, our data revealed that the HNF1β transcription factor that acts as a negative regulator for VDBP was upregulated, while the HNF1α transcription factor, the positive regulator for VDBP, was downregulated in low Vit D than in normal Vit D women after three or more RPL. This suggests that the HNF1β transcription factor may be critical in regulating VDBP levels and a potential therapeutic target for RPL [80]. However, our results indicate that VDBP, HNF1α, and HNF1β transcript levels are altered in women with RPL, even those with normal Vit D levels. This suggests that these changes may not be solely due to Vit D deficiency but could also be influenced by other factors associated with RPL, such as inflammation or oxidative stress. Alternatively, these changes may represent a compensatory mechanism to maintain normal Vit D levels in the presence of RPL. The dysregulation of HNF1β and VDBP expression in a low Vit D environment may have significant implications for reproductive health [81]. VDBP is essential for transporting Vit D to its target organs, including the placenta. Adequate Vit D levels are necessary for normal fetal development and preventing adverse pregnancy outcomes [82]. The observed upregulation of HNF1β and downregulation of HNF1α in low Vit D women after RPL suggests that this hormonal imbalance may contribute to the pathophysiology of infertility and pregnancy complications. Further research is needed to understand the underlying mechanisms and identify potential therapeutic targets.

The findings of this study highlight the importance of these specific residues in the binding between Vit D and HNF1β. The interaction is robust and likely stable, providing insights into these residues' potential functional roles. The involvement of ASN133 and ARG137 in the interaction suggests a possible regulatory role for HNF1β in the Vit D signaling pathway [83]. By modulating the activity of HNF1β through these interactions, 1,25-dihydroxy Vit D can regulate gene expression pathways related to calcium homeostasis and bone health [84]. Furthermore, these interactions contribute to the stabilization of the complex by enhancing the binding energy. The strong binding energy suggests that the complex is relatively stable and may play a role in the physiological functions of HNF1β and Vit D, and their interaction contributes to the overall stability and functionality of the complex.

This study has some limitations that are important to consider. The sample size, while adequate, could be more significant in enhancing the statistical power and generalizability of the findings. Additionally, the study was conducted in a specific region, and the participants were primarily of a particular ethnicity. Future studies with more extensive and diverse populations are recommended to validate these findings across different ethnicities and populations. The study's findings suggest that vitamin D supplementation may benefit women with RPL, but further research is needed to determine the optimal dosage and duration. Clinical trials are recommended to evaluate the efficacy of vitamin D supplementation in preventing RPL and improving pregnancy outcomes.

Future studies should investigate the relationship between Vit D deficiency, RPL, and the risk of chromosomal abnormalities, such as trisomy 18 and 21. This could involve cytogenetic analysis of the aborted fetuses to determine the prevalence of chromosomal abnormalities in women with RPL and different Vit D levels. In alignment with transcriptomic analysis, the current study focused on specific immunological and metabolic markers, so future studies could incorporate transcriptomic analysis to examine the expression of a broader range of genes involved in RPL. As per functional studies, future studies should also include functional assays to assess the impact of Vit D deficiency on immune cell function and metabolic pathways relevant to pregnancy. This could involve in vitro experiments using cell cultures or animal models to investigate the effects of Vit D supplementation on immune responses and metabolic parameters. By

addressing these limitations and pursuing these research directions, future studies can contribute to a deeper understanding of RPL and pave the way for improved prevention and treatment strategies.

Conclusion

To conclude, our results suggest a potential association between decreased Vit D levels and specific immunological and metabolic markers in women with RPL. RPL may be predisposed to increased risk among childbearing-aged women with failed clinical pregnancies. Maintaining a normal range of Vit D levels may help to reduce the risk of pregnancy complications and miscarriages; besides, the fetus has the relative potential to grow more rapidly, but further research is needed to confirm this association and determine the optimal levels of Vit D for pregnancy. Besides, our findings highlight the intricate relationship between Vit D status, adenosine metabolism, and purinergic signaling in the context of RPL. Further research is warranted to elucidate the underlying mechanisms driving metabolic dysregulation in RPL and to explore the therapeutic potential of targeting adenosine pathways and Vit D in mitigating the risk of recurrent miscarriages. Further, this study provides valuable insights into the molecular interaction between Vit D and HNF1β. The intense binding energy of -6.67 kcal/mol suggests a stable interaction, highlighting ASN133, LYS237, and ARG137 as crucial residues in the binding site of HNF1β. Identifying these residues indicates a role for HNF1β in the Vit D signaling pathway, warranting further investigation to explore its biological significance.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12884-024-06914-0.

Supplementary Material 1.

Acknowledgements
The authors extend their appreciation to the King Salman Center for Disability Research for funding this work through Research Group No. KSRG-2023-181.

Institutional review board statement
The University of Tabuk Ethics Committee approved the study by UT-367-204-2024, following the guidelines of the Helsinki Declaration.

Clinical trial number
Not applicable.

Informed consent statement
A written informed consent was obtained from all participants before the collection of blood sample.

Authors' contributions
Conceptualization, Aisha Nawaf Al balawi, Fuad Ameen, Alaa Elmetwalli, Riham Soliman; Formal analysis, Tarek El-Sewedy, Fuad Ameen, Nadia F. Ismail, Alaa Elmetwalli; Investigation, Alaa Elmetwalli, Noaf Abdullah N. Alblwi, Mervat G. Hassan, Tarek El-Sewedy; Project administration, Alaa Elmetwalli, Aisha Nawaf Al balawi, Fuad Ameen; Software, Alaa Elmetwalli, Ali H. El-Far, Noaf Abdullah N. Alblwi, Tarek El-Sewedy; Validation Alaa Elmetwalli, Aisha Nawaf Al balawi, Tarek El-Sewedy; Visualization, Alaa Elmetwalli, Nadia F. Ismail, Aisha Nawaf Al balawi, Fuad Ameen; Writing—original draft, Alaa Elmetwalli; Writing—review, editing, and provided critical feedback to help shape the research, analysis, and manuscript, Alaa Elmetwalli; All authors have read and agreed to the published version of the manuscript.

Funding
As part of Research Group No, KSRG-2023–181, the King Salman Center for Disability Research funded the research for this study.

Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 5 September 2024 Accepted: 21 October 2024
Published online: 29 October 2024

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