Perinatal changes in plasma of type 2 diabetes mellitus pregnancies who delivered newborns with cardiomyopathy, depression of the central nervous system, or hepatomegaly

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Abstract

Background

Maternal diabetes either pregestational or gestational is the main risk factor contributing in development of diabetic fetopathy (DF) in newborns. There are no generalized signs of DF up to late gestational age due to insufficient sensitivity of the currently employed instrumental methods for diagnosis.

Methods

This is a cross-sectional prospective controlled study. Here, we reported proteomic investigation for several cases of severe types of diabetic fetopathy (cardiomyopathy (CRDM, \(n = 37\)), central nervous system depression (CNSD, \(n = 35\)) and hepatomegaly (HPMG, \(n = 35\)) diagnosed during 30–35 gestational weeks and confirmed upon delivery by from patients with type 2 diabetes mellitus (T2DM). Control groups were comprised from women in whom T2DM had been ruled out \((n = 40)\) and group of pregnancies with T2DM who delivered healthy newborns \((n = 40)\).

Results

We found a composition of serum-based non-trivial markers capable that are strongly associated with the certain type of fetopathy or anatomical malfunctions in the affected newborns. Significant impact on mRNA splicing and DNA reparation has been determined by emerging alterations in CDCL5. Patients of CNSD groups were characterized by utmost depletion \((ca. 7\% of baseline)\) of DFP3 neurotrophic factor needed for the proper specialization of cardiomyocytes and oligodendrocytes. Corrupted regulation of non-canonical Wnt-signaling guided by PEDF (in CNSD and HPMG groups) and DAAM2 (in CRDM and HPMG groups) was also proposed. In addition, deficiency in retinoic acid and thyroxine transport was revealed by dramatic increase of TTHY in CNDS group.

Conclusions

We examined peripheral blood plasma and determined a small proportion of proteins indicating the pre-existing signs of DF. Most of the examined markers are participants of critical processes at different stages of embryogenesis and regulate various phases of morphogenesis. There are proteins regulating splicing and DNA repair, differentiation of neurons and their switching to the post-mitotic state. Therefore, reconstruction of the molecular interplay between the defined in proteins is decisive to appreciate cryptic violations in fetal development on the background of diabetic conditions.
Diabetic fetopathy (DF) is a severe complication defined in systematic changes of newborns and caused by maternal pre-existing (type 1 or type 2 (T2DM)) or gestational diabetes mellitus (GDM). Maternal hyperglycemia due to onset and duration of glucose intolerance during pregnancy may stipulate diabetic embryopathy resulted in congenital morphogenesis, endocrine impairments and spontaneous abortions [1]. DF can be pre-determined by ultrasound examination and Doppler scanning during pregnancy. However, due to insufficient sensitivity diagnosis of DF occurs in the late gestational age (beyond the 30th gestational week). A phenotypic analysis may yield the highest diagnostic value to reveal the grade of fetal hepatomegaly, cardiomyopathy, and pancreatic enlargement but the eventual results are reported upon delivery [2, 3].

The affected newborns may suffer metabolic syndrome, risk of obesity and respiration distress [1, 4]. Growing population of infants with metabolic syndrome as a consequence of maternal diabetic condition raises the great concern about cardiovascular disease developed already in adolescents [5]. The most prevalent sign of DF is macrosomia which is a response to maternal diabetes and can be considered as a highly valuable predictor for the later glucose intolerance and growing risk of improper fetal development. Up to 45% of infants born to diabetic mothers have a macrosomia which is almost 3 times higher than the rate comparing to normoglycemic patients [6].

Adipose tissue which produces adiponectin, TNF-α, IL-6, IL-10 and leptin, makes the main contribution in insulin sensitivity of pregnancies [7, 8]. While impaired maternal glycemic control and increased insulin resistance (IR), the level of circulating glucose is raised. The issue is that excessive maternal glucose may cross the placenta whereas insulin none. Starting from the second trimester, the fetal pancreas is already competent to secret insulin. Therefore, the combined condition of maternal hyperglycemia and excessive secretion of insulin in fetus engages the risk of increased fat production leading to macrosomia and malfunction of morphogenesis [6].

Heart defects are the most common type of diabetic fetopathy with prevalence up to 12% [9]. There is evidence arguing that exactly maternal increasing IR and high glucose level are mainly responsible for improper differentiation of cardiomyocytes and heart malformation [10]. Hypertrophic cardiomyopathy symptoms exhibited in asymmetric septal enlargement with a disproportionally hypertrophic septum were routinely found in 12.1% cases of infants affected by the maternal diabetes [10, 11].

Neural tube formation is also profoundly affected by maternal diabetic condition through direct influencing on regulation with a great variability of embryo genes expression, in particular, those involved in Wnt- and TGF-β signaling [12]. Recent studies also demonstrated that the etiology of neural tube defects in infants from diabetic mothers encompasses elevated levels of SOD (superoxide dismutase), inhibition of the pentose phosphate pathway (PPP) and extended oxidative stress [13, 14].

Hepatomegaly also holds the prevalent place of the potential infants' injury caused by maternal diabetes and in strict association with macrosomia [15]. It was proposed the great prognostic value of HbA1c for monitoring the risk of hepatomegaly however the indicator cannot be used solely [16]. Ultrasound
examination is still the most confident and reliable instrumental method for indication fetal hepatomegaly [17].

In this report, we provided a cross-sectional study among patients with T2DM who delivered newborns with different types of diabetic fetopathy. We focused on a proteomic assay and evaluated results for control group of non-diabetic pregnant women and patients with T2DM. Based on the obtained results, we performed a quantitative analysis of the small share of proteins and accommodated the most significantly altered markers in the reconstructed signaling pathways that determine morphogenesis, differentiation of cardiomyocytes, exhibit neurotrophic properties and activities toward mRNA splicing and DNA repair. It can be a correctly focused view that the majority of discovered mechanisms are generalized for all types of diabetic fetopathy in integrity, but there are small portion of specific markers that capable to segregate considered pathologies.

**Methods**

**Population**

In total, \(n = 187\) pregnant women at a gestational age of 30–35 weeks were participated in the study between October 2019–March 2020. Of them, \(n = 147\) patients had a history of T2DM for 7.6 ± 3.2 years and insulin therapy of 6.5 ± 2.5 IU/day. Patients were stratified according the type of diabetic fetopathy manifested in newborns upon delivery as follow: group with cardiomyopathy (CRDM; \(n = 37\), BMI = 28.78 ± 4.42 kg/m\(^2\); age 25.4 ± 4.2 years), depression of CNS (CNSD; \(n = 35\), BMI = 29.50 ± 3.21 kg/m\(^2\), age 25.9 ± 5.1 years) and hepatomegaly (HPMG; \(n = 35\), BMI = 29.78 ± 3.55 kg/m\(^2\), age 24.7 ± 4.7 years; Table 1). Patients with T2DM who ruled out the DF for newborns were combined in a separate group (\(n = 40\), BMI = 29.69 ± 4.13 kg/m\(^2\), age 25.4 ± 3.9 years; Table 1). Control group (\(n = 40\), BMI = 21.89 ± 4.29 kg/m\(^2\), age 25.6 ± 2.6 years; Table 1) comprised pregnancies who gave birth healthy newborns and had no previous history of T2DM or GDM according to the criteria of IADSPG (revision 2010) and adapted criteria of National Association of Obstetrician and Gynecologist (revision 2012).
Main anthropometric and clinical records for patients with T2DM and control group of patients. Patient with T2DM were subdivided into three groups according the signs of diabetic fetopathy attributed to the delivered newborns: CRDM – cardiomyopathy, CNSD – syndrome of central nervous system depression and HPMG – hepatomegaly. Patients with T2DM, who gave birth of newborns reported as healthy upon delivery and had no signs of diabetic fetopathy indicated by ultrasound examination during pregnancy course, were considered as the second control group.

| Group | T2DM | Control (healthy) | p-value |
|-------|------|-------------------|---------|
| Type of fetal complication | CRDM, n (%) | CNSD, n (%) | HPMG, n (%) | Healthy, n (%) |
| Size, n (%) | 37 (25) | 35 (24) | 35 (24) | 40 (27) | 40 | 0.889 |
| Age, years, mean ± SD | 25.4 ± 4.2 | 25.9 ± 5.1 | 24.7 ± 4.7 | 25.4 ± 3.9 | 25.6 ± 2.6 | 0.903 |
| BMI, kg/m², mean ± SD | 28.78 ± 4.42 (p = 0.021) | 29.50 ± 3.21 (p = 0.027) | 29.78 ± 3.55 (p = 0.015) | 29.69 ± 4.13 (p = 0.018) | 21.89 ± 4.29 | 0.273 |
| Family history of diabetes, n (%) | 10 (7) | 8 (5) | 8 (5) | 11 (7) | - | - |
| Duration of diabetes, years, mean ± SD | 7.8 ± 3.5 | 7.5 ± 4.2 | 8.0 ± 3.3 | 7.2 ± 4.1 | - | 0.739 |
| Fasting glucose level, mmol/L, mean ± SD | 7.8 ± 0.7 (p = 0.009) | 8.1 ± 0.7 (p = 0.003) | 7.6 ± 1.2 (p = 0.007) | 8.3 ± 0.6 (p = 0.004) | 3.5 ± 0.4 | 0.029 |
| Maternal weight gain, mean ± SD, kg | 10.7 ± 3.5 | 11.4 ± 2.8 | 11.2 ± 2.3 | 9.7 ± 4.1 | 9.1 ± 5.2 | 0.724 |
| Gestational age at delivery, days, mean | 272 | 275 | 272 | 274 | 270 | 0.951 |
| Caesar delivery, n (%) | 15 (10) | 19 (13) | 14 (10) | 18 (12) | 12 (30) | - |
| Fetal weight, g, mean ± SD | 3906 ± 212 | 3812 ± 229 | 3888 ± 197 | 3195 ± 231 | 3125 ± 132 | 0.031 |
| Apgar-1 score, median (range) | 8 (7–9) | 8 (6–8) | 8 (7–9) | 9 (6–9) | 9 (7–9) | 0.885 |
| Apgar-5 score, median (range) | 9 (8–10) | 9 (7–10) | 9 (8–10) | 9 (8–9) | 9 (8–10) | 0.963 |
| Diabetic fetopathy score, median (range) | 4 (2–7) | 3 (2–4) | 3 (1–5) | - | - | 0.093 |
| Hypoglycemia, n (%) | 11 (7) | 12 (8) | 8 (5) | 5 (3) | 2 (5) | 0.086 |

Diagnostic criteria of fetopathy
Phenotypic signs

Newborns were examined upon delivery according the following criteria: macrosomia if weight exceeded 4000 g; plentiful lanugo (soft auricles, soft bones of the skull, copious lubrication, undescended testes in the scrotum in boys); neonatal hypoglycemia if capillary glucose below 2.6 mmol/L.

Perinatal damage of CNS

Depression of CNS was examined as suppression of spontaneous motor activity, decreased passive and active muscle tone (decreased ability to straighten the body and head while holding the child by the shoulders, decreased tone of the flexors and extensors of the neck when transferred to sitting position), pathological eye symptoms, inhibition of Kussmaul's, Babkin's, and palmar grasp reflexes.

Fetal cardiomyopathy

Cardiomyopathy established by echocardiographic study: visualization of septal hypertrophy (left ventricular septal hypertrophy), thickness of interventricular septum was measured in M-mode in basal third if exceeded 1.8–4.5 mm for 1.5 h–1-year old infant.

Hepatomegaly

Signs recognized using echocardiographic study. Increased size of liver and the diameter of liver was measured by transverse scanning of the body to determine the distance between the most distant points of the right and left lobes. Craniocaudal size and anteroposterior size were determined during longitudinal scanning at mid-clavicular line.

Samples collection and handling

Samples preparation was performed according the protocol described in [18]. Briefly, following overnight fasting peripheral blood was collected into EDTA-2K tubes. Plasma obtained after centrifugation at 10°C and 2500 g for 10 minutes. Proteins concentration was measured using BCA assay kit. A total of 100 µg of each sample used for processing and enzymatic digestion with trypsin. Complete details of protocol are given in Appendix A.

High-resolution LC-MS analysis

Instrumental analysis was conducted as described in [18]. Briefly, the analysis was performed on a high-resolution Orbitrap Fusion (Thermo Scientific, Waltham, MA, USA) mass spectrometer equipped with a nano-flow NSI ions source and integrated with an Ultimate 3000RSLC (Thermo Scientific, Waltham, MA, USA) liquid chromatography system. Complete workflow and details are available in Appendix A.

Statistical analysis, quantitative estimation and functional annotation
Bias-correction to exclude outliers was performed by Mann–Whitney U-test at \( p < 0.05 \). Proteins shared between analyzed groups were normalized and ranged according their NSAF (normalized spectra abundancy factor) representation for quantitative analysis. Alterations between groups were represented for proteins in log-fold changes with significance cut-off \( p < 0.01 \) (Kruskal-Wallis test) and were obtained by defining a linear model for each protein. A two-sample moderated \( t \)-test used, and the reported \( p \)-values were corrected for false discovery rate. To extract proteins specific for each subgroup, we used PCA (principal component analysis) classification analysis, and the differences between scores were compared by Kruskal-Wallis test at \( p < 0.01 \).

To unveil biological processes and functions associated with meaningful alterations, proteins were submitted in Gene Ontology supported by the overrepresentation test (annotation release 20200407). Significance was estimated using Fisher’s exact test \( (p < 0.001) \) against the proteins identified in the control group, and Bonferroni correction applied for adjusting the FDR-based (false discovery rate) \( p \)-values \cite{19}. Initial (raw) molecular pathways were extracted from the KEGG \cite{20} and the Reactome (version 72) \cite{21}.

**Results**

**General description**

The assay was performed on 187 patients of whom 147 were pregnancies with a clinical history of T2DM for 7.6 years (average). Of them 37 patients gave newborns with cardiomyopathy (CRDM), 35 newborns with hepatomegaly (HPMG) and 35 newborns with syndrome of central nervous system depression (CNSD). Patients with T2DM did not differ significantly in BMI (Table 1) and fasting glucose level (7.8 mmol/L average, Table 1). Maternal weight gain was marginally higher in patients with T2DM compared to the control group, but the group of T2DM patients delivered healthy newborns was comparable to the baseline of the control group in this indicator (\( p = 0.694 \), Table 1). The proportion of patients with Caesar way of delivery was almost evenly distributed among T2DM subgroups and ranged from 10% (HPMG groups) to 13% (CNSD group) but the total rate was higher compared to the control group (45% against 30%, \( p = 0.007 \), Table 1). Although, patients with T2DM gave newborns with different types of diabetic fetopathy predetermined by sonography examination, the Apgar-1 and Apgar-5 scores reported upon delivery were somewhat comparable with scores reported in the control group (Table 1). The integrative score of diabetic fetopathy was distinctive between CRDM, CNSD and HPMG groups but the median value was indistinguishable throughout the study groups with antenatal complications (Table 1).

**Identification of proteins characterizing the condition of antenatal complications**

In total, we identified 378, of them 296 were shared throughout the study groups. Using Mann–Whitney U-test with the raw \( p < 0.05 \) and fold-change > 1.5 or < 0.8, only 42 proteins were determined as significantly altered and 30 proteins may have a meaningful value to discriminate T2DM subgroups (healthy and different types of fetopathy under consideration, Table 1 and Appendix B). The significant compounds
with $p < 0.05$ were passed to PCA, with PC1 explaining 18% variance, and a PC2 of 11%. PCA score discrimination analysis showed satisfied segregation of T2DM patients who gave birth healthy newborns from the control group, and good separation and dispersion of subgroups bearing signs of diabetic fetopathy from the control group and from T2DM patients with healthy newborns (Fig. 1A). This indicates a meaningful impact of diabetic condition during gestation on the possible risk and complications of fetal morphogenesis.

At the same time, it was established that only $n = 20$ proteins among 42 can be accounted as significant (with $p < 0.01$) for distinguishing groups of antenatal pathologies (CRDM, CNSD and HPMG) from groups of T2DM patients who gave birth healthy newborns and the control group. This indicates different contribution of the selected proteins in way of antenatal complications. Significantly differed proteins ($n = 17$) were endorsed by the Kruskal-Wallis test with fold changes $>2$ and $<0.5$ (linear scale) and $p < 0.01$. These proteins were selected for clustering into a heatmap exhibiting a different pattern between the studied groups in respect of antenatal pathologies (Fig. 1B and Table 2).
The most significantly altered proteins (n = 17) segregate types of diabetic fetopathy from the control group and T2DM patients who gave birth of healthy newborns. Fold-changes are represented as the median in a logarithmic (log\(_2\)) scale. False discovery rate adjusted p-values reported in the last column was obtained using Bonferroni correction for multiple testing. Significant fold changes are color filled with in the table.

| Recommended protein name (according to the UPKB nomenclature) | Primary gene name | FC Log (2) values | p-value |
|---------------------------------------------------------------|-------------------|-------------------|---------|
| Pigment epithelium-derived factor                             | SERPINF1          | CNSD: 1.103       | HPMG: 0.600 | CRDM: 0.314 | < 0.001 |
| Thrombospondin-1                                              | TSP1              | CNSD: 1.394       | HPMG: 0.558 | CRDM: 0.471 | 0.004   |
| Zinc finger protein DPF3                                       | DPF3              | CNSD: -3.833      | HPMG: 0.051 | CRDM: 1.033 | 0.005   |
| Complement C4-A                                               | CO4A              | CNSD: 0.539       | HPMG: -0.131 | CRDM: 0.044 | 0.002   |
| Complement C1q subcomponent subunit C                         | C1QC              | CNSD: 0.543       | HPMG: 0.483 | CRDM: 0.043 | 0.003   |
| Transthyretin                                                  | TTR               | CNSD: 0.655       | HPMG: 0.398 | CRDM: 0.471 | 0.006   |
| Complement C1q subcomponent subunit B                         | C1QB              | CNSD: 0.674       | HPMG: 0.118 | CRDM: -0.437 | 0.003   |
| Actin, cytoplasmic 1                                           | ACTB              | CNSD: 0.744       | HPMG: -0.103 | CRDM: -0.634 | < 0.001 |
| Cell division cycle 5-like protein                            | CDC5L             | CNSD: 1.254       | HPMG: -0.068 | CRDM: 0.198 | 0.003   |
| Complement C5                                                  | CO5               | CNSD: 1.403       | HPMG: 0.499 | CRDM: 0.393 | 0.004   |
| Serum amyloid P-component                                     | APCS              | CNSD: 0.943       | HPMG: -0.418 | CRDM: 0.269 | < 0.001 |
| Disheveled-associated activator of morphogenesis 2            | DAAM2             | CNSD: 0.145       | HPMG: 0.734 | CRDM: 1.068 | 0.004   |
| Laminin subunit beta-4                                         | LAMB4             | CNSD: 0.320       | HPMG: -1.085 | CRDM: 1.664 | 0.002   |
| Neutrophil defensin 1                                          | DEF1 *            | CNSD: -0.480      | HPMG: -1.058 | CRDM: -0.197 | 0.006   |
| Platelet basic protein                                         | CXCL7 *           | CNSD: 0.201       | HPMG: 0.775 | CRDM: 0.046 | 0.006   |
| N-acetylmuramoyl-L-alanine amidase                            | PGLYRP2 *         | CNSD: 0.296       | HPMG: 1.023 | CRDM: 0.428 | 0.004   |
| Complement C1r subcomponent                                   | C1R               | CNSD: -0.607      | HPMG: 0.107 | CRDM: 0.542 | 0.005   |
| Complement C2                                                  | CO2               | CNSD: -0.147      | HPMG: 0.127 | CRDM: 0.936 | < 0.001 |
| Leucine-rich alpha-2-glycoprotein                             | LRG1              | CNSD: 0.369       | HPMG: -0.004 | CRDM: 0.724 | < 0.001 |
| MKL/myocardin-like protein 2                                   | MKL2              | CNSD: -0.073      | HPMG: 0.372 | CRDM: 0.798 | 0.006   |
Some proteins among the selected are shared between any two types of fetopathy under consideration and showed significant changes (for example, PEDF (pigment epithelium-derived factor) and TSP1 (thrombospondin-1) for CNDS and HPMG groups; or DAAM2 (disheveled-associated activator of morphogenesis 2) and LAMB4 (laminin subunit β4) for HPMG and CRDM groups, Table 2) whereas most of the determining markers demonstrated specificity to the certain type of antenatal complication and did not attain a relevance to cognate groups. Probably, such segregation can be underlined by engaging of different mechanisms deployed during CRDM, HPMG or CNSD (Appendix C).

Functional categorization of the identified proteins

Most listed proteins (Table 2 and Appendix B) related to biological processes of regulation of response to stimuli (GO:0048583, FDR = 6.19e-05) and to stress (GO:0080134, FDR = 0.00034) with an average local clustering coefficient of 0.575 (PPI enrichment p < 1.0e-16). Biological GO terms stratification supported upregulation of proteins related to defense response (GO:0006952, FDR = 3.54e-07), regulation of inflammatory response (GO:0050727, FDR = 1.03e-05) and response to glucose (GO:0009749, FDR = 0.0025). As expected, an overwhelming majority of the detected proteins (n = 25 of 30, Appendices B and C, and Table 2) were attributed to localization in extracellular space (GO:0005576, FDR = 2.57e-13) and secretory granule lumen (GO:0034774, FDR = 2.41e-07). Most of proteins revealed peptidase regulatory activity (GO:0061134, FDR = 0.0037), oxygen binding and carry activity (GO:0005344, FDR = 0.0074) and general activity guiding and regulating molecular function (GO:0098772, FDR = 0.0282). Analysis of biochemical reactions and transformations revealed general signaling pathways defined to innate immune response (HSA-168249, FDR = 5.52e-06), hemostasis (HSA-109582, FDR = 0.0015) and amyloid fibers formation (HSA-977225, FDR = 0.00029).

We purposed to assemble the detected proteins into molecular network that can disclose differences between types of diabetic fetopathy (CRDM, CNSD, and HPMG) and can determine triggers that severely underline findings of fetopathy from patients with T2DM.

Protein-protein interactions (PPI)

Protein-protein interaction (PPI) analysis was conducted on n = 30 proteins capable to discriminate T2DM patients from subjects with uncomplicated pregnancy (Appendix C). The assayed set completely covers n = 17 most significant markers that segregate groups of patients with T2DM who delivered newborns with different types of diabetic fetopathy (CNSD, CRDM and HPMG, Table 2).

There are n = 9 common (adjacent) markers between three groups of diabetic fetopathy: APOC4 (apolipoprotein C-IV), LG3BP (galectin-3-binding protein), PEDF, TSP1, PHLD (phosphatidylinositol-glycan-specific phospholipase D), ALS (insulin-like growth factor-binding protein complex acid labile subunit), DPF3 (zinc finger protein DPF3), DAAM2 and LAMB4. PPI analysis showed that these proteins produce a poorly dense core of functional interactions (the coefficient of protein interactions is PPI = 0.389), except for a pair of PEDF and TSP1 and cluster of DAAM2, DFP3 and LAMB4. Assumingly, it indicates involvement of these proteins in the general processes leading to the development and progression of a
particular fetopathy. Given the large number of groups of specific markers, most likely, adjacent markers are a reflection of secondary signs of fetopathy, and encompass peripheral biological process. The multiple molecular functions possessed by adjacent markers also support this proposition; these proteins are characterized by pleiotropic property in the cell's life cycle. It is worth noting that one of the most important proteins is LAMB4, since this protein is a direct participant in the organization of tissues and the process of organogenesis in the embryonic period and seems critical for determining cell migration and proper orientation in the processes of organogenesis and tissue formation, also fixing cells in the intercellular matrix. Similar functions are performed by TSP1, which is paired with PEDF, but its role is largely limited to the adhesive functions that determine the intercellular interaction and the interaction of cells with the extracellular space.

Three functional clusters (Appendix C) are formed by proteins involved in the immune response, proteins involved in the structuring of the intercellular space, and regulatory signaling proteins. Regulatory function is accomplished through the expression of proteolytic catalytic activity, as well as through the transmission of signal (ligands) to receptors along signaling pathways, thereby forming a network of humoral regulation of the immune response and the body's protective response (GO: 0006952, \( p = 0.00025 \)). The vast majority of the proteins participating in the interactions (17 proteins) are secreted proteins, or proteins whose main activity is manifested in the extracellular space region (GO: 0044421, \( p = 7.90e-09 \)), which indicates their high para- and endocrine potentials.

**Discussion**

The impact of mRNA splicing and DNA reparation during embryogenesis

We focused on the most important markers, which can be either cross-specific for several types of fetopathy or highly specific like CDC5L (cell division cycle 5-like protein; Table 2). According to the functional analysis, CDC5L generates a local core of interactions through ACTB (actin, cytoplasmic 1), DEFA1B (neutrophil defensin 1), and C2 (complement factor C2) with the local clustering coefficient 0.782; the last two elements (DEFA1B and C2) are not accounted as CNSD-specific (Table 2). According to the data of Human Protein Atlas, CDC5L is characterized by low tissue specificity, but its highest expression is traced in brain cortex, cerebellum, hippocampus and thalamus.

It is difficult to overestimate the role of CDC5L in regulation of cell division, DNA repair and activity guiding mRNA splicing and maturation. Recent data highlighted the association of CDC5L with neuronal differentiation during embryogenesis, the implementation of which occurs through the assembly of active complex (CDC5L/14-3-3β/PRP19α) carrying necessary competencies for differentiation of neurons [22]. Phosphorylation of PRP19α (pre-mRNA-processing factor 19) by Akt-kinase is a critical step in complex assembly, whereupon it acquires a conformational ability to bind with 14-3-3β for translocation into the nucleus (Fig. 2) and to meet CDC5L. Obviously, dysregulation of one of these elements leads to an adverse influence on neuronal differentiation and corrupts cell division, since the complex regulates G2/M phase.
The relationship between CDC5L and some neurodegenerative and neuroimmune diseases is established in plenty researches, most of which operate with transcriptome analysis [23, 24, 25]. Adverse regulation of mRNA splicing in cerebral cortex through, inter alia, malfunction of CDC5L lead to a decrease of proliferative activity and disruption of the neuronal differentiation [26]. Comparable results were obtained on HeLa after immunoprecipitation of a complex of CDC5L with PLRG1 (pleiotropic regulator 1) with heteronuclear ribonucleoprotein (hnRNP) necessary for the activity of mRNA splicing complex (Fig. 2). Mutant cells (CDC5−/−) displayed inability to build the complex, disruption of splicing process and underwent extensive apoptosis [27].

Evidence exist that translation repression and degradation of mRNA is accomplished by circulating miRNAs tightly associated in different complexes [28]. Recent GWAS (genome-wide associated study) data demonstrated that about 600 different miRNAs are expressed in placenta and play a key regulatory role in embryogenesis and metabolic adaptation during fetal growth. Such molecules are released in maternal circulation and may indicated cautions of placenta dysfunction and GDM [29]. Our proteomic data suggests that increased concentration of CDC5L (Table 2) may reflect a potential malfunction of mRNA splicing caused by a dysregulation in placental miRNA providing instructions for mRNA processing.

DNA repair is another important aspect of CDC5L activity. Embryonic fibroblast cells undergo active apoptosis through the p53-mediated mechanism in animals deficient in PLRG1 [30]. The fundamental is a decreased ability of PLRG1 to assemble with CDC5L and consequent abolishment of DNA repair mechanism (Fig. 2). As has been highlighted, such embryos demonstrated failed cells division and fragmented nuclei and die rather early due to apoptosis and re-localization of CDC5L from nucleus to cytoplasm (Appendix C). Repeated experiments on cultivating cardiomyocytes and neurons showed similar results, where, apart the re-localization of CDC5L, a decrease of the apoptosis regulator Bcl-2 was noted [30, 31]. Murine heterozygous by CDC5L demonstrated a survival rate not exceeding 25%, but the survived embryos characterized by a dramatic increase of degraded neurons and cardiomyocytes (Appendix C) which was accompanied with a reduced Bcl-2 and increased level of the phosphorylated p53 but unaltered Bax [30].

Hence, CDC5L is an important regulator of mRNA splicing and DNA repair in differentiating neurons during embryogenesis. Therefore, drastically increased CDC5L in CNSD group (Table 2) may suggest possible dysregulation of placental miRNA and the ongoing accumulation of DNA errors caused by oxidative stress typical for patients with the manifesting T2DM.

Extended activation of the immune response

Due to a high perception to changes in homeostasis and immune response, elements of complement system are typically accounted as non-specific markers although some of them were meaningful in both CNSD (complement factors C1QC, C5, C4, C1QB) and CRDM (complement factors C1B, C2) groups (Table 2). As outlined above, in patients with manifesting T2DM activation of complement system is
associated with oxidative stress caused by hyperglycemic state [32]. Overproduction of ROS (reactive oxygen species) is aggravated by accumulated blood glucose undergoing auto-oxidation (Fig. 2), which activates synthesis of prostaglandins and deployment of inflammatory reaction. A significant contribution is caused by activation of FASN (fatty acids synthase) and, consequently, inclined consumption of NADPH through the enhanced rate of PPP (pentose-phosphate pathway) and boosted G6PDH (glucose-6-phosphate 1-dehydrogenase) [33].

As level of ROS inclines, the rate of TGFβ-mediated signaling enhances toward overproduction of cytokines (IL1, IL6, IL10, TNFα) and including complement factors [32, 34]. The accumulated ROS and RNS (reactive nitrogen species) positively affect NF-kB pathway that triggers NOS (NO-synthase) and entails to NO overproduction positive regulation of prostacyclin (PTGIS). Unfortunately, extremely high concentration of NO returns to reaction with ROS and generation of dangerous peroxynitrites (Fig. 2) [33].

Hence, it highlights an odd situation when, on the one hand, there is excessive NO generation, and on the other hand, limited access to NO due to reacting with ROS. In turn, overproduction of cytokines stimulates activation and overproduction of compliment system elements, which are well recognized in the study (Table 2).

Repressed transition from progenitor to post-mitotic state.

Transcriptional activation and repression of selected genes by chromatin remodeling is a key mechanism guiding tissue and organogenesis where DPF3 takes the role of an important indicator (Table 2). Evidences suggest that the maximum expression of DPF3 is observed in hypothalamus, neurons, myoblasts and oocytes. Its expression is regulated through Mef2, which recognizes for DFP3 promoter with high specificity [35]. As has been overviewed on the cultivated HEK293 cells and later on myoblasts, soon after switching DFP3 gene off, development and differentiation of cells are aborted [36].

Neurogenesis and myogenesis (including cardiac and skeletal muscle) are orchestrated by DFP3, but the exact mechanism of action is still uncertain. The tacit assumption is action through the binding to certain acetylated and methylated regions of histones, thought, direct participation in the regulation of chromatin remodeling. Accordingly, global estimation of methylome has been proposed as a potential hallmark for the assessment of gestational diabetic conditions and its dire consequences affecting fetal development [29]. Notable, DPF3 is a necessary element of nBAF complex (neuron-specific chromatin remodeling complex BRG1/BRM associated factor), and upon completion of the progenitor activity of neurogenic cells, the essential for proliferative capacity of npBAF (neural stem/progenitor cells) complex (ACTL6A/BAF53A and PHF10/BAF45A) is replaced by a neuron-specific complex comprised homologous ACTL6B/BAF53B and DPF3/BAF45C.

Some studies outlined that significantly increased DPF3 expression leads to cardiac hypertrophy during embryogenesis, while the same was also shown in adults with chronic cardiac hypertrophy [37]. In both occasions, the process of hypertrophy is associated with activation of embryo-specific genes, including DPF3a and DPF3b isoforms, and with reprogramming the expression of β-MHC and skeletal actin genes.
Other report indicated that initiation of Hirschsprung's disease is largely due to depletion of DFP3 gene, which causes a decrease in cell migration to the appropriate localization of nerve ganglia in various parts of the intestine, especially, in stenotic segment. [38].

One can note the close interaction of DPF3 with RUNX (runt-related transcription factors) signaling attendant with TGF-β route and increasing proliferative rate [39, 40]. The RUNX regulator LRG1 (leucine-rich alpha-2-glycoprotein) is located upstream and its increased expression repeatedly reported in context of various diseases due to dysregulation of the TGF-β pathway. However, in our study increased expression of LRG1 is noted only in the CRDM group (Table 2).

Both CRDM and CNSD groups were distinguished in DFP3, but CNSD characterized by the lowest abundance (Table 2). Our data suggest a distinctive feature of DFP3 in neuron-specific association with chromatin remodeling being executed during embryogenesis and dramatically dropped in the CNDS group. In CRDM group upregulation of LRG1 and DFP3, assumingly, reflects the impaired proliferation where DFP3 is the final point (switching to the post-mitotic state) and LRG1 is the starting point, thereby determining expression of RUNX factors and, consequently, TGF-β pathway.

Regulation of myelinization and differentiation of cardiomyocytes via competing for canonical and non-canonical Wnt-signaling

A neurotrophic factor PEDF was found as an element taking an active part in genesis of neurons and cardiomyocytes (Table 2). It exhibits pronounced properties of angiogenesis inhibitor and belongs to a serine protease but does not undergo characteristic stress/relaxed conformational changes. The highest expression of PEDF is tracked in retina, thalamus, dendrites, and all neurons irrespective of their topology and adaptation; latest data also suggests expression of PEDF in liver cells [41].

Implication in regulation of differentiation intends PEDF to interact with ECM (extracellular matrix) proteins and proteins responsible for the architecture of organelles and cytoskeleton. Among those TSP1 is the most engaging adhesive glycoprotein intended for establishing the cells-to-extracellular matrix connection and modulation CD36-mediated angiogenesis (Table 2). The crew of PEDF and TSP1 shows strong implications in the regulation of Wnt-signaling (p = 0.01594, Fig. 3).

Assumingly, PEDF acts as an endogenous inhibitor of Wnt/β-catenin pathway through exposure to LRP6 (low-density lipoprotein receptor-related protein 6) co-receptor. The expression level of PEDF increases significantly in response to Wnt3a which employs LRP6 as a co-receptor for signal transmission [42] (Fig. 3). Simultaneously, it decreases in response to non-canonical Wnt5a-induced signaling depleted on utilization of LRP6. Matching results were obtained using siRNA when direct interaction of PEDF with LRP6 (K_d=3.7 nM) was abolished forwarding of signal transmission via Wnt-pathway [43]. The mechanism, demolishing ligand (Wnt3a) with receptor (LRP6) dimerization, is accomplished through the reduction of LRP6 phosphorylation by PEDF. (Fig. 3). Apparently, inhibition of canonical Wnt-signaling affects down-streaming elements, including suppression of TSP1 necessary for angiogenesis and
associated morphogenesis via TGF-β signaling. Suppression of TSP1 is manifested in 25% decrease of 4-hydroxyproline needed for collagen synthesis and structuring of ECM.

Hence, assumingly that the base of competing experience between PEDF and TSP1 is explained by their antagonizing relationship during angiogenesis. Both proteins characterized by the straight functional interaction but by the inversed regulation: while CNSD group was featured by down-regulation of PEDF and up-regulation of TSP1, the situation looked counter in CRDM group; and HPMG group characterized by the increased TSP1 (Table 2). Thus, comprehensive regulation during embryogenesis is tailored to the compensatory mechanism due to activation of non-canonical signaling. This mechanism requires, instead, Wnt5a and tyrosine-protein kinase transmembrane receptor ROR2, thereby translocating β-catenin to the nucleus and keeping its level essential for signal transduction. [44, 45].

Considering the enhanced rate of non-canonical Wnt pathway, one cannot ignore an extremely important DAAM2 protein readily recognized (frequency > 0.8) in HPMG and CRDM groups (Table 2). DAAM2, as a switcher, governs the non-canonical Wnt [46] (Fig. 4). It is an utmost regulator for various processes during embryogenesis including determination of myelination, development of spinal cord, potentiation and clustering of signalosome. There are two isoforms exist, and both, DAAM1 (disheveled-associated activator of morphogenesis 1) and DAAM2, are necessary for the development of myocardial cells and maturation of sarcomeres through the organization of actin cytoskeleton [47]. Disruption of DAAM2-mediated transduction leads to cerebral amyloid angiopathy, degeneration of epithelial basement membrane and various oncogenic manifestations [48, 49].

Non-canonical Wnt-signaling is not a substituent of the abolished canonical one, but rather, a cognate pathway bypassing β-catenin activation [50] and necessary for the controlling of sympathetic neurons morphogenesis, determination of cell specialization, migration and polarization [51, 52, 53]. It was found that during differentiation of peripheral axons from embryonic fibroblasts, Wnt5a binds to ROR2 co-receptor providing phosphorylation of DAAM2 which is prerequisite for the signal transmission [50, 51] (Fig. 4).

As evidenced, knockdown of Wnt5a by siRNA dramatically decreases or completely abolishes DAAM2 phosphorylation [54]. Other reports demonstrated that inhibition of canonical Wnt pathway does not depend on ROR2 co-receptor, since its absence or blocking retains the ability of Wnt5a and DAAM2 to switch and potentiate the non-canonical one [55, 56].

Impaired expression of DAAM2 leads to non-compaction cardiomyopathy (NCC). At least in murine models, animals with excessive or, on the contrary, suppressed expression of DAAM1 or DAAM2 were characterized by the failure nucleation of actin fibrils in embryonic fibroblasts, excessively deep trabeculae myocardial cavities and left ventricular NCC (Appendix C) [57]. Cardiomyocytes looked round and smaller size with a weak interconnection due to inhibited secretion of collagen and decreased rate of actin polymerization (Appendix C). Later, it was found that the molecular function of DAAM2 is caused by the FH1 domain necessary for capping of actin filaments, and by the FH2 domain supporting the binding and assembly of actin subunits [58]. Malfunction of DAAM2 is manifested in lack or weakly articulated Z-
bands of sarcomeres and anatomically disorganized $M$, $H$ and $I$ bands [57, 59] which becomes of growing attention in regard of the extremely increased DAAM2 in CRDM group (Table 2).

The activity of DAAM2 is involved in regeneration of myelin sheath of oligodendrocytes which is inhibited after white matter injury [60]. The process is regulated via activation of DAAM2-PIP2 pathway to achieve complex between PIP5K (phosphatidylinositol 4-phosphate 5-kinase) and DAAM2 (Fig. 4), and downstreamed to excite PLR5/6 receptors [60, 61]. Here, the role of DAAM2 is restricted to regulation of oligodendrocytes differentiation and axonal myelination, thereby increased DAAM2 expression during embryogenesis and in adults leads to depression of myelination. In this respect, CNSD was the only group with unaltered abundance of DAAM2 in our research (Table 2). Trials with DAAM2$^{-/-}$ animal embryos reported about considerably increased degree of axonal myelination and weakly controlled enhanced differentiation of oligodendrocytes [62]. Nevertheless, hypoxia condition promotes activation of myelination, which suggests a negative effect on DAAM2-PIP5K axis in regulation of Wnt signaling [62].

Assumingly, regulation of Wnt signaling is achieved through the competitive binding with LPR6 receptors, whereas binding to ROR2 receptors is necessary for interplay with DAAM2 that encourages launching of morphogenesis, myelination and immune response through potentiating of NF-kB signaling (Fig. 4).

Overexpression of DAAM2 during pregnancy places developing fetus at a risk of cardiomyopathy and irregular tissue development.

Coupled retinoic acid deficiency and thyroxine transport bring forth the deployment of oxidative stress

Transthyretin (TTHY) is a secreted protein with affinity for thyroxine an order of magnitude greater than for triiodothyronine which makes him the main carrier of thyroid hormone. Besides, TTHY has an extraordinary function for transporting of retinoic acid (RA). It binds with two molecules of RBP (retinol binding protein) in stable complex thereby reducing the glomerular ultrafiltration of RA and stabilizing the complex of RA with RBP [63]. Normally, up to 40% of circulating TTHY is bounded with RBP, while the rest act as a carrier of thyroxine (Fig. 5).

The most prevalent disease associated with TTHY is transthyretin amyloidosis, which occurs due to inability to congregate in regular stable homotetrameric structure and, hence, to transport thyroxine and RA to sites of their further transformation [64]. Clinical findings are accompanied by formation of manifested amyloid fibrils due to the accumulation of unstructured TTHY oligomers [65] close to ventricular septum and synaptic region of large neurons [66] causing various types of neuropathy and cardiomyopathy [67].

Serum concentration of TTHY in newborns is varied between 50–200 µg/mL, and rapidly increases over twice in the adults. In patients with severe cardiomyopathy, the concentration of circulating TTHY and fragments is about 250 µg/mL due to chronic transthyretin amyloidosis [68, 69]. In patients with neurodegenerative diseases, the concentration and the expression level are increased in order of magnitude [70]. The acceptable range of TTHY is wide enough (69–650 µg/mL) and may vary in response to dietary preferences and immune response, therefore, it is rather difficult to appreciate its
diagnostic significance [70, 71]. Nevertheless, admittedly that the normal concentration of TTHY in blood is 200–400 µg/mL and its half-life is 2 days [68].

The abundance of TTHY directly correlates with estradiol which increases significantly during pregnancy. Blocking of estradiol or cortisol receptors is exhibited in significant decreasing of circulating TTHY [72]. According to our data, TTHY is up-regulated specifically in CNSD group assumingly caused by both expectedly increased estradiol and deficient in RA (Table 2).

There are data on the latent proteolytic activity of TTHY, when the protein cleaves β-amyloid into small fragments incapable to aggregate into amyloid plaques [73]. Therefore, relationship between TTHY, lipids metabolism and transport of RA becomes imperative. Up to 2% of TTHY are in complex with APOA1 (apolipoprotein A-I), which exhibits antioxidant activity (Fig. 5). An increased concentration of circulating TTHY due to the raised uncoupling with RBP primes exhibition of proteolytic activity largely focused on APOA1. Recently, combination of the substantially decreased APOA1 with BMI in patients with T2DM and GDM was considered as a risk factor for prediction of diabetic condition [74, 75]. Fragments of APOA1 aggregate into apolipoprotein amyloid fibrils and ultimately causes inflammation, activation of compliment system and augmented oxidative stress [76].

Hence, a deficiency of RA can provoke undesirable consequences as a result of increased concentration of circulating TTHY and consequential declination of APOA1. However, overproduction of NO in T2DM patients also accelerates oligomerization of TTHY through its S-nitrosylation [77] that foster to the extension of oxidative stress (Fig. 5).

Conclusion

In this study, we assumed different mechanisms guiding the morphogenesis out of proportion toward different type of fetopathy. However, obviously, the key issue is emerged from the diabetic condition during pregnancy. Assumingly, that depending on personal clinical history, the outcome of progressing diabetic conditions on the growing fetus may vary, but the grade of influence can be vague and outcome in cardiomyopathy, neuropathy of liver enlargement, or combination of several types fetopathy. It is hard to propose the final outcome without clinically relevant and robust markers using only currently employed instrumental methods despite their efficiency.

Most of the examined markers are participants of critical processes at different stages of embryogenesis and regulate various phases of morphogenesis. There are proteins regulating mRNA splicing and DNA repair, differentiation of neurons and their switching to the post-mitotic state, implication in Wnt pathway guiding tissue morphogenesis and critical for the proper specialization of cardiomyocytes and oligodendrocytes. All these markers can be distinguished in plasma samples of pregnancies suffering diabetic condition. Therefore, reconstruction of the molecular interplay between the defined in proteins is decisive to appreciate cryptic violations in fetal development on the background of diabetic conditions.
Abbreviations

ACTB - actin, cytoplasmic 1;
ALS - insulin-like growth factor-binding protein complex acid labile subunit;
APOA1 - apolipoprotein A-I;
APOC4 - apolipoprotein C-IV;
C2 - complement factor C2;
CDC5L - cell division cycle 5-like protein;
CNSD - central nervous system depression;
CRDM – cardiomyopathy;
DAAM1 - disheveled-associated activator of morphogenesis 1;
DAAM2 - disheveled-associated activator of morphogenesis 2;
DEFA1B - neutrophil defensin 1;
DF - diabetic fetopathy;
DPF3 - zinc finger protein DPF3;
ECM - extracellular matrix;
FASN - fatty acids synthase;
FDR - false discovery rate;
G6PDH - glucose-6-phosphate 1-dehydrogenase;
GDM - gestational diabetes mellitus;
GO – gene ontology;
hnRNP - heteronuclear ribonucleoprotein;
HPMG – hepatomegaly;
IADSPG - The International Association of the Diabetes and Pregnancy Study Groups;
IR - insulin resistance;
LAMB4 - laminin subunit β4;
LG3BP - galectin-3-binding protein;
LRG1 - leucine-rich alpha-2-glycoprotein;
LRP6 - low-density lipoprotein receptor-related protein 6;
nBAF - neuron-specific chromatin remodeling complex BRG1/BRM associated factor;
NCC - non-compaction cardiomyopathy;
NOS - NO-synthase;
npBAF - neural stem/progenitor cells remodeling complex BRG1/BRM associated factor;
NSAF - normalized spectra abundancy factor;
PCA - principal component analysis;
PEDF - pigment epithelium-derived factor;
PHLD - phosphatidylinositol-glycan-specific phospholipase D;
PIP5K - phosphatidylinositol 4-phosphate 5-kinase;
PLRG1 - pleiotropic regulator 1;
PPI – protein-protein interaction;
PPP - pentose-phosphate pathway;
PRP19α - pre-mRNA-processing factor 19;
PTGIS – prostacyclin;
RA - retinoic acid;
RBP - retinol binding protein;
RNS - reactive nitrogen species;
ROR2 - tyrosine-protein kinase transmembrane receptor ROR2;
ROS – reactive oxygen species;
RUNX - runt-related transcription factors;
T2DM - type 2 diabetes mellitus;
TSP1 - thrombospondin-1;
TTHY – transthyretin;

Declarations

Statement of Ethics

The study design was approved by the local Ethical Committee of the Perinatal Center at N.E. Baumann 29th Hospital (Moscow; local protocol identifier BAU-EP2019-R035.B02 on October 15, 2019). All handlings and use of material were provided according the WMA Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects (revision 2013).

Consent for publication

This paper does not contain any personal data in any form. All subjects gave their signed written consent to participate in the study. Consent of participation and data personal data security were obtained according the National Legislation of the Russian Federation "On Personal Data" No. 152 of July 27, 2006 (refer to official government link to acquire: http://www.kremlin.ru/acts/bank/24154). All subjects participated in the study were anonymized. Authors complied ethical standards and privacy policy and did not disclose any personal data of participated subjects in the paper or in the Supplementary materials in any form.

Availability of data

Supplemental materials are available along with this article: Appendix A – details of sample preparation protocol and LC-MS analysis; Appendix B – details for the most significantly altered proteins among study groups and general description of newborns upon delivery; Appendix C - details of statistical analysis, protein-protein interaction analysis and commentaries to CDC5L and DAAM2 proteins.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

Competing interests

The authors declare no conflict of interest.

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Authors contribution

O.P., I.G. and G.K. responsible for samples collections, clinical assay and involved in samples preparation; L.K., E.S. and T.M. contributed in selection of patient meeting the clinical and investigation requirements, design of experiment and data overview; L.K., M.N.K. and A.K. involved in samples preparation and processing and participated in data analysis; A.L.K, A.T.K, and S.G.M contributed in design of experiment, data analysis and interpretation, manuscript draft and final version writing.

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