Introduction
Neural tube defects (NTDs) are caused by inadequate closing of the neural tube. Alpha-fetoprotein has limitations due to its poor sensitivity and specificity in NTD detection. Proprotein convertase subtilisin/kexin type 9 (PCSK9) appears to have an escalating role in neurogenesis. This study aimed to evaluate the two forms of PCSK9 (Mature PCSK9 and furin cleaved) circulating in NTD maternal sera as a potential novel non-invasive biochemical marker for prenatal screening of NTDs.

Materials and Methods: In this case-control study, the presence of PCSK9 in serum samples of 30 pregnant women with current NTD fetuses (case group) and 30 pregnant women with a healthy singleton pregnancy (control group) was evaluated by Western blot analysis followed by the immuno-precipitation approach.

Results: Median of mature PCSK9/furin ratio among the case group was 0.92 (0.05-2.54) versus 1.29 (0.19-6.62) among the control group ($P = 0.02$). Receiver operating characteristic curve analysis for mature PCSK9/furin ratio displayed a sensitivity and specificity equal to 63.3%.

Conclusions: The ratio of mature PCSK9 to furin cleaved form was significantly reduced among women with current NTD fetuses compared to women having healthy pregnancies. This ratio can be a potential original biochemical marker in the non-invasive prenatal screening of NTDs.

Keywords: Sensitivity, Specificity, Neural tube, Screening, Fetus

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Introduction
Neural tube defects (NTDs) are critical congenital deformities in the central nervous system (CNS) (1). Throughout fetal development, neural tube development is an essential process (2). If a partial neural tube closing occurs, its neuro- epithelium is now bare to the amniotic fluid leading to its disintegration and neuron insufficiency (3). Various gene-environment influences trigger the occurrence of NTDs (4). The clinical approach for prenatal screening regarding various congenital anomalies started around 50 years ago by measuring the above range for alpha-fetoprotein (AFP) in amniotic fluid among pregnant females with current NTD fetuses (5). Raised AFP level in the maternal serum is a screening tool for NTDs, usually done at 15 weeks of gestation. Yet, there are some concerns regarding AFP sensitivity and specificity (6). AFP used as a screening tool is influenced by numerous factors and is restricted by its high false-positive rate (7). AFP is in direct correlation with the pregnancy weeks at the time of prenatal visit, body mass index (BMI), race, smoking, and parity (8,9). Raised AFP was also seen in association with other bad pregnancy outcomes. For example, preeclampsia, intrauterine fetal death, preterm birth as well as oligohydramnios (10). Around 95% of NTD cases occur unexpectedly in females with a short family history of NTDs. This highlights the urgency of prenatal screening to detect NTDs (11). NTDs can be discovered by ultrasound (12). However, its accuracy is usually affected by fetal position, maternal BMI, operator skills doing the ultrasound (13).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) was primly recognized in cerebellar neurons as an mRNA which is highly expressed during the progression of apoptosis (14). It is member number nine in the cluster of serine proteinases. It slices nonfunctioning secretory precursors and changes them to functioning proteins and peptides. PCSK9 gene is positioned on chromosome 1p32.3 (15). The proprotein convertase enzyme (furin) slices mature PCSK9. Furin deactivated PCSK9. Although it is lower in concentrations, the cleaved form flows in the circulation and intact form (16).

PCSK9 regulates the physiology of the CNS in many different ways, mainly through neuronal differentiation and metabolism of the low-density lipoprotein (LDL) receptor family. PCSK9 is poorly expressed in the human brain, but PCSK9 expression rises inside the human brain in coexistence with the neurological disease. There is a strong link between PCSK9 found in Cerebral spinal
fluid and NYDs (17). It was also reported that PCSK9 levels were lesser in NTD maternal serum than the sera of healthy pregnant women by 0.73 fold (7). We aimed by this work to evaluate two forms of PCSK9 (Mature PCSK9 and furin cleaved) circulating in NTD maternal sera as a potential novel non-invasive biochemical marker for prenatal detection of NTDs.

Materials and Methods

Study Setting and Participants

In this case-control study, 60 pregnant women referred to the Recurrent Pregnancy Loss Clinic, Prenatal Diagnosis and Fetal Medicine Department, National Research Centre, Cairo, Egypt, from June 2019 to January 2021 were enrolled and followed up till delivery in two groups: 30 pregnant women with current NTD fetuses as the case group and 30 pregnant women with a healthy singleton pregnancy as the control group. All data were recorded from the participants’ medical files.

Pregnancy with an NTD fetus was diagnosed by screening tests at 13-22 weeks of gestation and confirmed by the ultrasound. Follow-up was performed for the low-risk women to determine whether their fetuses were normal or not. All low-risk women that NTD was not detected by ultrasound scan in their fetus were excluded from the study. We identified all fetuses for NTDs using prenatal ultrasound examination via General Electric Voluson P8 real-time scan system to measure gestational age and evaluate viability. A full anomaly scan was done to assess any anomalies in fetuses among the case group. The control group was selected from the pregnant women with a healthy singleton pregnancy that had no any congenital anomalies, history of NTDs, bad pregnancy outcomes, or complicated pregnancies”. All participants took folic acid regularly under doctor supervision during pregnancy.

Collection of Serum Samples

Three milliliters of peripheral blood samples was taken from all women (weeks 13-22). The collection and handling of the gathered serum were done following operational procedure to decrease pre-analytical disparity. Serum was separated from peripheral blood samples directly through centrifugation at 4°C at 4000 r/min for 5 minutes, then extra centrifugation at 14 000 r/min for 5 minutes at 4°C to get rid of any cellular remains. Storage of serum aliquots was done at -80°C.

Analysis for CSPK9 Using Western Blot

Immunoprecipitation of PCSK From Plasma

Thirty microliters serum was incubated with 10 μg PCSK9 antibody at 4°C for an hour on a rotating shaker (Thomas Scientific, United States). 20 μL of resuspended protein A/G agarose beads (Santa Cruz, Germany) were introduced to the collected serum at 4°C for an hour on a rotating shaker. Samples were centrifuged for 15 minutes at 2000 rpm and 4°C. Supernatant existed was thrown away, and the beads were swept away using 5X using phosphate buffered saline. The pellets were resuspended in electrophoresis buffer and boiled for 2 minutes. Subsequently, centrifugation (Sigma Co., Germany) was done for 10 minutes at 2500 rpm at 4°C, and 10 μL aliquots were used for electrophoresis.

Immunoblotting of PCSK9 Protein

The loading of different samples was done on 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gel. Following electrophoresis, proteins were placed on nitrocellulose membranes with pores diameter 0.45 um (Amersham). The membranes were saturated for 30 minutes with 5% fat-free milk in Tris-buffered saline with 0.1% Tween 20 detergent. The membranes were incubated with PCSK9 antibody (Santa Cruz, Germany) in dilution 1:200. Anti-mouse secondary antibody was used in dilution 1:5000. Proteins were detected by chemiluminescent revelation (Invitrogen Novex ECL, Thermo Fisher, United States), and the bands were detected by Charge-Coupled Device camera (Azure c280). Western blot is less likely to give false-positive results. Therefore, western blotting has higher specificity, making it independent regarding antibody specificity (18).

Data Analysis

Statistical analysis was handled by means of IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical statistics were stated as mean and standard deviation (SD) or in the form of median and range as properly. Qualitative statistics were stated in the form of frequency as well as percentage. Fisher exact test was done to test the correlation among qualitative variables. As for quantitative statistics, an independent sample t test was used to compare two groups. A receiver operating characteristic (Roc curve) was used to find a cutoff off value regarding mature CPSK9, furin, and mature CPSK9/furin ratio band intestines of the western blot to be able to promptly classify subjects normal or reduced for the protein concentration (18) and P value was obtained by chi-square test. Accuracy was expressed using sensitivity, specificity, the likelihood ratio of a positive test, and the likelihood ratio of a negative test. P value < 0.05 was considered significant.

Results

A total of 60 pregnant women were enrolled in the study
in two case and control groups (n=30/each). There was no significant difference in the mean of age between two study groups. The consanguinity rate among the case group was higher than the control group as well as BMI (Table 1). The demographic characteristics of the participants are summarized in Table 1. The NTD fetuses among the case group were included anencephaly (n=2), meningocele (n=16), and Spina bifida (n=12).

Serum PCSK9 immunoprecipitation followed by western blotting revealed the presence of 2 PCSK9 forms (Mature and furin-cleaved). The two forms were detected as two bands at approximate molecular weights 70 kDa for the mature PCSK9 and 55 kDa for the furin-cleaved form, as shown in Figure 1. The median of band intensities for mature PCSK9 and furin-cleaved showed no statistical significance between the case and control group, however the ratio of mature/furin showed a statistical significance between the case group and the control group (Table 2).

ROC curve analysis was done for band intensities of mature PCSK9 and furin cleaved forms. It demonstrated cut-off values (26226 and 18705), which are the optimum threshold to predict NTD (Figure 2a, 2b). For mature PCSK9 and furin cleaved the ROC curve displayed sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) as shown in Table 3, Figures 2 and 3. Among the case group, there were 20 NTD pregnancies with a reduced expression for mature PCSK9. In comparison, only 12 healthy pregnancies (control group) had reduced expression of mature PCSK9. Only 13 NTD pregnancies among the case group had reduced expression of the inactive form (furin cleaved) compared to controls. Only 18 healthy pregnancies had shown reduced expression of the inactive form (Table 4).

Among case group, median of mature PCSK9/furin ratio was statistically significant (in comparison to control group). ROC curve demonstrated a cut-off value of 0.985 (Figure 2b). With a sensitivity and specificity, PPV, and NPV demonstrated in Table 3. 11 women among the case group expressed a high normal mature CPSK9/furin cleaved ratio. Whereas, 19 women among the control group expressed a high normal mature CPSK9/furin cleaved ratio (P = 0.039) (Table 4, Figure 3).

**Discussion**

NTDs are hypothetically a multifactorial disorder. Due to large number of genetic mechanisms as well as other environmental influences, it is not conceivable to rely only on one biomarker for diagnosing NTDs. That is why PCSK9 protein was selected for this study. PCSK9 is exceedingly found in the brain during embryonic development.

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**Table 1. Demographic Characteristics of the Participants in the Two Studied Groups (n=30/each)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Case Group</th>
<th>Control Group</th>
<th>P Value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y), mean ± SD</td>
<td>26.9 ± 4.6</td>
<td>26.7 ± 3.8</td>
<td>0.87</td>
</tr>
<tr>
<td>BMI (kg/m(^2)), mean ± SD</td>
<td>28.6 ± 5.2</td>
<td>24.3 ± 3.7</td>
<td>&lt;0.001(^b)</td>
</tr>
<tr>
<td>Live births (No. of children), mean ± SD</td>
<td>2±1</td>
<td>2 ± 1</td>
<td></td>
</tr>
<tr>
<td>Consanguinity (1(^st) degree relative), No. (%)</td>
<td>11 (36.7)</td>
<td>4 (33.3)</td>
<td>0.03</td>
</tr>
<tr>
<td>Family history for NTDs, No. (%)</td>
<td>5 (16.7)</td>
<td>3 (10)</td>
<td>0.70</td>
</tr>
<tr>
<td>Obstetric history, No. (%)(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HELLP syndrome</td>
<td>3 (0.9)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Preeclampsia</td>
<td>4 (1.2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Abortion</td>
<td>13 (3.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IUGR</td>
<td>1 (0.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Preterm delivery</td>
<td>3 (0.9)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gestational diabetes</td>
<td>6 (1.8)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

BMI: Body mass index; NTDs: Neural tube defects; HELLP: Hemolysis, Elevated Liver enzymes and Low Platelets; IUGR: Intrauterine growth retardation.

\(^a\) Independent sample t test.

**Table 2. Molecular Weight of Band Intensities of (Mature CPSK9, Furin Cleaved, Ratio of Mature CPSK9/Furin) Among Both Studied Groups**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Case Group</th>
<th>Control Group</th>
<th>P Value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature CPSK9</td>
<td>20220.9 (683.0-48746.0)</td>
<td>29039.4 (5533.0-46294.0)</td>
<td>0.06</td>
</tr>
<tr>
<td>Furin cleaved</td>
<td>21668.3 (2895.41-58536.2)</td>
<td>19595.7 (4278.8-88936.2)</td>
<td>0.40</td>
</tr>
<tr>
<td>Ratio mature/furin</td>
<td>0.92 (0.05-2.54)</td>
<td>1.29 (0.19-6.62)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

CPSK9: Proprotein Convertase Subtilisin/kexin type 9

Data presented as median (range).

\(^c\) Mann Whitney test.
growth stages and is highly expressed in cerebellar neurons. It regulates the physiology of the CNS in many different ways, mainly through neuronal differentiation. It was documented in a previous study that PCSK9 was simultaneously poorly expressed among sera of pregnant rats with NTDs in comparison with pregnant rats in the control group (7). PCSK9 is involved in many biological actions such as lipid breakdown and absorption, cellular apoptosis, inflammation reactions, neuronal formation as well as tumor metastasis (19). It is important among the process of LDL-receptor breaking down inside liver cells as well as endothelial tissue. Atypical lipid profiles, precisely LDL are related with poor pregnancy outcomes. Therefore PCSK9 presence is involved in the cholesterol metabolism which is needed in the neuronal methylation processes during embryogenesis (20).

It had been stated that furin cleaved PCSK9 have a much less action or role in regulating LDL receptor (LDLR) as well as serum LDL cholesterol in comparison to mature PCSK9 (15, 21, 22). Therefore, it is vital to quantity both forms of PCSK9 independently to be able to identify the importance of mature PCSK9 as well as furin-cleaved PCSK9 in cases of NTDs. Though there is not a precise method that has been described for measuring furin-cleaved PCSK9, therefore assessing the ratio of each form of PCSK9 with NTDs would aid in increased sensitivity and specificity of the test.

Our study evaluated the two forms of PCSK9 (Mature PCSK9 and furin cleaved) circulating in NTD maternal sera. Our results revealed no difference in expression of mature PCSK9 between women with current NTD pregnancies compared with women carrying healthy fetuses. Mature PCSK9 expression was reduced among NTD pregnancies compared to the control group but did not reach statistical significance. As for furin cleaved form, it was highly expressed among cases compared to the control group but also did not reach statistical significance.

### Table 3. Area Under Curve Analysis of Mature CPSK9, Furin Cleaved, and Mature CPSK9/Furin Ratio

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature CPSK9</td>
<td>66.7</td>
<td>60.0</td>
<td>62.5</td>
<td>64.3</td>
<td>63.3</td>
</tr>
<tr>
<td>Furin cleaved</td>
<td>43.3</td>
<td>60.0</td>
<td>52.0</td>
<td>51.4</td>
<td>51.7</td>
</tr>
<tr>
<td>Ratio mature/Furin</td>
<td>63.3</td>
<td>63.3</td>
<td>63.3</td>
<td>63.3</td>
<td>63.3</td>
</tr>
</tbody>
</table>

PPV: positive predictive value; NPV: negative predictive value; CPSK9: Proprotein Convertase Subtilisin/Kexin type 9.

Data presented as percentages.

### Table 4. Comparison of Mature CPSK9, Furin Cleaved, and Mature CPSK9/Furin Ratio in the Two Studied Groups (n=30/each)

<table>
<thead>
<tr>
<th></th>
<th>Case Group</th>
<th>Control Group</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature CPSK9, No. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>10 (33.3)</td>
<td>18 (60)</td>
<td>0.38</td>
</tr>
<tr>
<td>Reduced</td>
<td>20 (66.7)</td>
<td>12 (40)</td>
<td></td>
</tr>
<tr>
<td>Furin cleaved, No. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>17 (56.7)</td>
<td>12 (40)</td>
<td>0.79</td>
</tr>
<tr>
<td>Reduced</td>
<td>13 (43.3)</td>
<td>18 (60)</td>
<td></td>
</tr>
<tr>
<td>Mature/furin cleaved ratio, No. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High normal</td>
<td>11 (36.7)</td>
<td>19 (63.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Low normal</td>
<td>19 (63.3)</td>
<td>11 (36.7)</td>
<td></td>
</tr>
</tbody>
</table>

Chi-square test.
However, we took a further step by measuring the ratio of mature PCSK9 to furin cleaved from among the two studied groups. We found out that the ratio was reduced among the case group compared to the control group. It was statistically significant, having a $P = 0.02$ (Table 2) and a sensitivity and specificity of 63.3% (Table 3). Even more, 19 women having current NTD pregnancies expressed a low mature CPSK/furin ratio compared to only 11 women with healthy pregnancies who expressed a low mature CPSK/furin ratio; $P = 0.039$ (Table 4). To expand more understanding regarding those biochemical findings, we tried to interpret the reason behind why pregnant women carrying NTDs had a decreased ratio of mature PCSK9 to furin cleaved in comparison to women carrying healthy fetuses from the control group; it is hypothesized that furin is the key PCSK9 active protease inside the cells (23). Observing the SDS-PAGE, the cleaved PCSK9 demonstrates a modification in 70 kDa band, converting it to a lower 55-kDa which does not have Ser153-Arg218 amino acid site of the catalytic. Therefore we assumed that those pregnant women carrying NTD fetuses and were statistically significant for reduced mature PCSK9/furin ratio might be affected by this modification which leaves the furin cleaved PCSK9 unable to engage to the LDL receptor (16) and consequently affecting closure processes of the embryo’s neural tube. Furin cleaved form of PCSK9 circulates in human plasma (16,23), forming 40% of the entire flowing PCSK9 (23). PCSK9 is deactivated by furin (24). Furin cleavage sequence 215RFHRQ219 mediates some gains of function mutations (25,26). There is also a possibility that those pregnant women carrying NTD fetuses with the reduced mature PCSK9/furin ratio might be carrying one of those mutations, affecting the PCSK9 furin cleavage processes (16,23,25), signifying that furin antagonism is the primary molecular cause leading to gain of function phenotype. Whether or not furin cleavage of PCSK9 disturbs receptor engagement and post ligation measures is still under study. For instance, if cleaved PCSK9 is naturally inactive but still preserved to bind to the LDL receptor normally, the cleaved PCSK9 found in the circulation could act as a competitive inhibitor to the intact form mature PCSK9.

There is extremely a scarce in the literature regarding the correlation of PCSK9 with NTDs. Most of the published literature focus on other neurological diseases (27). However, Dong and his colleagues examined the possible correlation between PCSK9 expressions among serum females encountering NTDs pregnancy. The study revealed that PCSK9 is greatly reduced among pregnant women carrying NTD compared to women carrying healthy fetuses in the control group by 0.73-fold decrease. Their study also documented that PCSK9 had evidences of analytic efficacy with AUC = 0.763, making it a possible biomarker in cases of NTDs, having a sensitivity of 56.67% and specificity of 98%. They also found that PCSK9 level among spinal cords among healthy rat fetuses is progressively augmented over the embryo’s growth period. Nevertheless, PCSK9 expression level among spinal cords and placentas was dramatically lowered in the NTDs rat fetuses compared to normal rat fetuses (7). Earlier ontogeny research in mouse embryos revealed that PCSK9 is briefly found in the cerebellum throughout brain growth. It is also revealed that it is mainly found in the extended rostral part of the olfactory peduncle throughout growing up. Those findings show that PCSK9 might exist in critical stages of neuron synthesis, immigration, differentiation of neural cells. PCSK9 surge among primary neuronal cultures increases the variation of cortical neurons (28). Another study had revealed that PCSK9 is found in human CSF (29). Lately, it was stated that PCSK9 released in the CNS is maximally found in the cerebellum throughout perinatal growth. It is expressed in high brain levels among grownups after the ischemic attack (30). Our study is also the first to validate the Western Blot method to measure both PCSK9 forms; mature PCSK9 and furin-cleaved form. This method is likely to be valuable for examining the physiological or pathological activity of PCSK9. The limitations that faced our study were the lack of national standardization of data regarding the incidence of NTDs despite the widespread of NTD cases among our population. Note to worth the scarcity in published literature regarding this topic.

**Conclusions**

Highlighting the important role of cleaved PCSK9 could improve the possibility of early detection and prediction of pregnancies carrying NTDs in the upcoming years. Larger prospective, comparative studies are needed to confirm the validity of CPSK9 as a potential novel biomarker for the non-invasive prenatal screening of NTDs and its clinical significance.

**Authors’ Contribution**

MMS apprehended the idea for the review and its structure, reviewed literature and drafted the manuscript. TAS apprehended the idea for the review, recruitment of participants, and drafted the manuscript. SS lab work up as well as prepared gel electrophoresis figure for publication, critical revision of the manuscript for important intellectual content and draft the manuscript. All authors approved the final version of the manuscript.

**Conflict of Interests**

The authors have no conflict of interest to declare.

**Ethical issues**

The study proposal was approved by the Medical Ethical Committee of National Research Centre (NRC), Cairo, Egypt (Code: 19266). In accordance with the Helsinki Declaration, a written informed consent form was signed by all participants after a full explanation of the purpose and steps of the study.

**Financial Support**

This study had no financial support.

**Acknowledgments**

We would like to thank all recruited women who cooperated us to get this study done.
1. Shaker et al. References

Shaker et al. work is properly cited.


