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Assessment of The Level of Protein C in Children with Nephrotic Syndrome

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ABSTRACT

Background: thromboembolic events remain one of the most serious complications in patients with nephrotic syndrome and this thrombotic tendency could be related to deficiency in natural coagulation inhibitors.

Objective: the aim of our study was to estimate P-C quantity &quality in the blood of nephrotic patients for the possible role that it might play in the pathogenesis of thromboembolic complications in these patients & trial to find correlations between P-C & some hemostatic parameters.

Patient and Methods: this study was carried out on 60 children (age: 3-13years) were diagnosed as nephrotic patients. They were classified as follow: G1- comprised of 20 cases in relapse, G2- comprised of 20 cases in remission (the same cases of relapse), G3- comprised of 20 healthy children as control group.

Results: Our study showed very high significant increase in P-C, both in activity and concentration in relapse but in remission its level decreased towards the normal level but still slightly increased and this increase is statistically non-significant. As regard serum albumin and serum total protein, the both were very highly significantly increased in relapse and remission as compared to the control. As regard PT and PTT were found to be highly significant decreased in relapse as compared to the control group. **Conclusion:** from the foregoing we can concluded that, Increase level of P-C in N.S. denoted that it has no role in thromboembolic complications and Increase level of P-C in N.S may afford some protection against the thrombotic diathesis by counteracting hypercoagulability state.

Keywords: Protein C, Children, Nephrotic syndrome.

INTRODUCTION

The haemostatic mechanism comprises a series of integrated reactions of the vessel wall, platelets, plasma coagulation and fibrinolytic factors directed towards prevention of blood loss from intact vessels & to arrest bleeding from injured vessels. Variation in any of these factors may lead to either excessive bleeding or thrombosis[(1)].

Protein c is a vitamin K-dependent protein of 62 kDa that circulates in blood as a zymogen in a concentration of about 4 µg/ml. The protein is synthesized in the liver. The mature protein C molecule is composed of a light and a heavy chain .The two chains being disulfide-linked. The light chain consists of a Gla domain and two epidermal growth factors (EGF)-like domains. The heavy chain contains a short activation peptide and a serine protease domain. Similar to the procoagulant co-factors, protein C circulates as an inactive proenzyme and needs to be activated to gain anticoagulant activity[(2)].

Protein C is activated by thrombin bound to thrombomodulin on the surface of endothelial cells. Activation is more efficient when protein C binds to the endothelial cell protein C receptor (EPCR) via its Gla domain. Activated protein C (APC) is a physiologic anticoagulant that down-regulates thrombin generation by cleaving and inactivating factor Va and factor VIIIa, the cofactors of the prothrombinase and intrinsic Xase complexes, respectively; protein S acts as a cofactor for APC-mediated inactivation of factor Va and factor VIIIa by helping target APC to the negatively charged surface of activated platelets[(3)].

The activity of protein C may be down-regulated by reducing the amount either of available thrombomodulin or of EPCR. This may be done by inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), Protein C may be up-regulated by platelet factor 4. This cytokine is conjectured to improve activation of protein C by forming an electrostatic bridge from protein C's Gla domain to the glycosaminoglycan (GAG) domain of thrombomodulin, reducing the Michaelis constant (KM) for their reaction[(4)].

Nephrotic syndrome is a common renal disorder seen in childhood. In African & Indian children, the incidence appears to be higher, ranging from 11 to 16 per 100.000. The nephrotic syndrome is characterized by Oedema, massive proteinuria, hypoalbuminaemia, and hyperlipidaemia. The serum albumin is usually 2.5 gm/dL or less. There is elevation of the plasma cholesterol early, with increase in the triglycerides when the serum albumin falls to about lgm/dL[(5)].

Once the diagnosis of N.S has been established, the next step is to determine its etiology. In children, approximately 90 per cent result from a primary glomerulopathy, with only 10 per cent being secondary to a systemic disease. Renal biopsy is indicated in those children who present with atypical features of the N.S. & in those with frequent relapses and severe steroid toxicity where cytotoxic therapy is considered[(6)].

The underlying pathogenetic abnormality in nephrosis is proteinuria, which results from an increase in glomerular capillary wall permeability[(7)]. Infection is the major complication of nephrosis; it results from

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increased susceptibility to bacterial infections during relapse. Thromboembolic events remain one of the most serious complications in patients with nephrotic syndrome. Edema is the one of greatest concern to both patients & their parents[8]. The child may be hospitalized with the first episode of nephrosis for diagnostic, educational, and therapeutic purposes. When edema develops, sodium intake is reduced. Remission is then induced by administration of prednisone. If there are repeated relapses, cyclophosphamide therapy should be considered[9]. Most children with steroid responsive nephrosis will have repeated relapses until the disease resolves itself spontaneously toward the end of the 2nd decade of life[10]. Thromboembolic events remain one of the most serious complications in patients with nephrotic syndrome. The reported incidence of thromboembolic episodes in people with nephrotic syndrome ranges from 6% to 44% and it has been ascribed to the presence of a hypercoagulablestate[11].

AIM OF THE WORK

The aim of our study is to estimate P-C quantity &quality in the blood of nephrotic patients for the possible role that it might play in the pathogenesis of thromboembolic complications in these patients & trial to find correlations between P-C & some hemostatic parameters

PATIENTS AND METHODS

I- Patients: The present work included 40 children, were diagnosed as nephrotic syndrome patients. They were chosen from Pediatric Department & Pediatric Out-Patient Clinic, New Damietta, Al-Azhar University. Twenty healthy children were taken as a control group.

The study was approved by the Ethics Board of Al-Azhar University and an informed written consent was taken from each participant in the study.

They were classified into 3 groups:

1- Group 1 (Patients in Relapse):

This group comprised 20 patients already diagnosed as nephrotic syndrome in relapse (whatever the etiologies, the underlying pathologies & the treatment given). Their ages ranged from 3-13 years; they were 17 males & 5 females.

2- Group 2 (The same patient in group 1 in Remission State):

3- Group 3 (control Group): 20 healthy children were chosen as a control group. Their ages ranged from 3-13years, they were 8 males & 2 females.

Inclusion criteria:

1- Age: 3-13 years old.
2- Gender: both sexes.
3- Patients fulfilling the diagnosis of nephrotic syndrome.

Exclusion criteria:

1- Age: Less than 3 years or older than 13 old.
2- Any other chronic disease e.g. Liver, Cardiac……etc.
3- Nutritional edema.

II- Methods:

All patients & controls were subjected to the following:

- **Full history**: Including personal history, presenting compliant and past history of similar condition.
- **Clinical examination**: Including general and local examination.
- **Laboratory investigations:**

The laboratory investigations which were performed in the present work were divided into Routine investigations and Specific investigation.

Sampling:

For all subjects, morning urine sample was taken for albumin to creatinine ratio and blood samples were withdrawn. About 5 ml of blood were withdrawn with minimal stasis.

Blood was divided into tubes as follows:

1. One ml was carried into tube containing K-EDTA for (CBC).
2. 9 parts of blood to one part of sodium citrate (3.8%) in a plastic tube & gently mixing by inversions after capping then was centrifuged within 10 minutes of collection; the separated plasma was divided into two capped tubes, one stored at -70°C until assayed for protein c and the other used for coagulation profile.
3. The rest of the blood was delivered into a plain tube, left to clot and then was centrifuged within 10 minutes of collection; the separated serum was used for routine investigation.

All laboratory investigations were done at Al-Azhar University hospital (New Damietta), Clinical Pathology department research laboratory. The routine investigations were carried out on the same day of collection of blood NB: Lipemic, icteric and hemolyzed specimens were discarded.

Routine investigations

- Complete blood counting (CBC): by automated blood counter (Sysmex XS-5100).
- Serum creatinine, Serum Albumin, Serum total protein, ACR in urine, Serum Cholesterol and Serum Triglyceride measured by fully automated chemistry analyzer (Bechman Coulter AU 480).
- Coagulation profile (PT-APTT): by automated coagulometer (Coatron –M).

Specific investigations:

1- Quantitative Determination of Protein C in plasma by ELISA Technique

Principle: -

Protein C kit provides reagents for the qualitative determination of protein C in plasma by the sandwich technique of enzyme immunoassay known as ELISA. A plastic support (microwell) coated with specific rabbit antiprotein C antibody is allowed to come into contact with plasma containing the protein C to be measured. The protein C contained in the plasma binds to the plastic support by one of its antigenic determinants. Next, rabbit

anti-protein C antibody coupled with peroxidase is added, and this binds to the free remaining antigenic determinants of protein C, forming a sandwich. The bound enzyme peroxidase is then revealed by its activity in a predetermined time on the substrate orthophenylenediamine (OPD) in the presence of hydrogen peroxide. The colour change that is produced is a direct measure of the PC concentration initially present in the plasma.

Sample for Testing: Citrated plasma as mentioned before and diluted 1 : 50 with dilution buffer.

Statistical analysis

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc, Chicago, Illinois, USA). Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage.

The following tests were done:

- Independent-samples t-test of significance was used when comparing between two means.
- Chi-square (x^2) test of significance was used in order to compare proportions between two qualitative parameters.
- The confidence interval was set to 95% and the margin of error accepted was set to 5%. The p-value was considered significant as the following:
- Probability (P-value):
- P-value <0.05 was considered significant.
- P-value <0.001 was considered as highly significant.
- P-value >0.05 was considered insignificant.

RESULTS

Table (1): Comparison between patient group & control group as regards demographic data

		Patient group		Control group		Chi square test	
		No	%	No	%	X^2/t*	P value
Sex	F	10	25.0%	5	25.0%	0.000	1.000
	M	30	75.0%	15	75.0%		
Age	Mean ±SD	8.15	3.21	8.30	3.16	-0.172*	0.864

* Independent t test

Table (**1**) shows that there was no statistically significant difference in demographic data regarding studied group.

Table (2): Comparison between patient group & control group as regards Laboratory investigations

	Patient group		Control group		Independent t test	
	Mean	SD	Mean	SD	t	P value
Albumin	2.68	0.89	4.50	0.24	-8.903	0.001
Total Protein	5.51	0.90	7.12	0.45	-7.502	0.001
Alb/Creat. Ratio -Urine	2577.50	2384.07	18.05	6.67	4.781	0.001
Creatinine	0.75	0.20	0.75	0.16	-0.082	0.935
C3 (complement)	123.13	34.26	115.20	17.59	0.970	0.336
Cholesterol	221.98	77.38	112.75	13.33	6.241	0.001
Triglyceride	150.58	43.95	81.75	11.84	6.853	0.001

Table **(2**) shows that there was statistically significant increase in patient group in comparison to control group with Albumin/Creatinine Ratio, Cholesterol, Triglyceride, white blood cells and statistically significant decrease in patient group in comparison to control group with Albumin and Total Protein.

Table (3): Comparison between remission stage and relapse stage & control group as regards laboratory investigations

	Control group		Remission stage		Relapse stage		One way ANOVA	
	Mean	SD	Mean	SD	Mean	SD	f	P value
Albumin	4.50	0.24	3.53	0.19	1.83	0.29	602.429	0.001
Total protein	7.12	0.45	6.15	0.57	4.87	0.69	75.818	0.001
Albumin /Creatinine Ratio	18.05	6.67	257.00	117.58	4898.00	562.70	1373.976	0.001
Creatinine	0.75	0.16	0.75	0.16	0.75	0.24	0.013	0.987
C3	115.20	17.59	118.85	18.59	127.40	45.01	0.878	0.421
Cholesterol	112.75	13.33	149.65	31.69	294.30	16.52	379.647	0.001
Triglyceride	81.75	11.84	109.80	13.25	191.35	17.02	321.240	0.001

This table shows that there was statistically significant decrease in relapse group with Albumin and total protein while statistically significant increase in relapse stage with Albumin /Creatinine Ratio, Cholesterol and Triglyceride

Table (4): Comparison between remission stage and relapse stage & control group as regards Haemostatic investigations

	Control group		Remission stage		Relapse stage		One way ANOVA	
	Mean	SD	Mean	SD	Mean	SD	f	P value
PT	14.03	0.57	13.61	0.95	10.96	0.60	104.296	0.001
PTT	38.52	2.29	35.60	3.43	29.55	6.00	23.644	0.001
PLTs	239.35	17.72	237.25	15.61	236.00	14.09	0.227	0.797

This table shows that there was statistically significant decrease in relapse group with PT, PTT.

Table (5): Comparison between remission stage and relapse stage & control group as regards PC.a and PC.c

	Control group		Remission stage		Relapse stage		One way ANOVA	
	Mean	SD	Mean	SD	Mean	SD	f	P value
PC (conc.)	3.56	0.46	3.89	0.63	4.45	0.59	12.777	0.001
PC (active)	119.65	13.42	130.20	17.04	139.10	14.52	8.351	0.001

This table shows that there was statistically significant increase in relapse stage with PC.a and PC.c.

Table (6): Comparison between remission stage and relapse stage & control group as regards Haematological investigations.

	Control group		Remission stage		Relapse stage		One way ANOVA	
	Mean	SD	Mean	SD	Mean	SD	F	P value
MCV	77.95	4.16	74.45	4.21	59.10	5.23	96.658	0.001
MCH	26.55	1.07	25.96	2.41	22.30	2.56	23.494	0.001
HB	13.01	2.44	12.69	0.48	9.94	0.72	25.59	0.001
WBCs	5.98	1.19	6.33	0.76	10.63	5.85	11.15	0.001

This table shows that there was statistically significant increase in relapse group with WBCs while statistically significant decrease with MCV, MCH and HB.

Table (7): Comparison between remission stage and relapse stage & control group as regards family history and ASC

		Control group		Remission		Relapse		Chi square test	
		No	%	No	%	No	%	X^2	P value
F.H	A	20	100.0%	13	65.0%	13	65.0%	9.130	0.010
	P	0	0.0%	7	35.0%	7	35.0%		
Ascites	A	20	100.0%	20	100.0%	10	50.0%	24.000	0.001
	P	0	0.0%	0	0.0%	10	50.0%		

This table shows that there was statistically significant increase in remission and replex with family history and ascites.

Table (8): Correlation between PC.c and PC.a as regards age and other variables in control group:

	PC.c		PC.a	
	R	P value	R	P value
Age	0.02	0.932	-0.153	0.520
Albumin	-0.002	0.995	0.129	0.587
Total Protein	-0.071	0.767	-0.165	0.488
lbumin /Creatinine Ratio	0.051	0.83	0.149	0.530
PT	-0.361	0.118	-0.489	0.059
PTT	0.054	0.821	-0.03	0.900
Creatinine	0.301	0.198	0.281	0.231
C3	-0.443	0.050	-0.446	0.049
Cholesterol	0.351	0.130	0.369	0.109
Triglyceride	0.089	0.710	0.175	0.46
WBCs	0.218	0.356	0.116	0.627
HB	0.331	0.154	0.178	0.454
PLT	0.117	0.622	0.056	0.813
MCV	0.177	0.455	0.01	0.967
MCH	0.201	0.394	0.048	0.842

This table shows that PC.c and PC.a has no correlation with all parameters in control group.

Table (9): Correlation between PC.c and PC.a as regards age and other variables in remission stage:

	PC.c		PC.a	
	R	**P value**	**R**	**P value**
Age	0.194	0.412	0.094	0.692
Albumin	-0.924	0.001	-1.000	0.001
Total Protein	-0.687	0.001	-0.821	0.001
lbumin /Creatinine Ratio	0.355	0.125	0.512	0.021
PT	-0.570	0.009	-0.505	0.023
PTT	-0.031	0.897	-0.003	0.990
Creatinine	0.470	0.037	0.372	0.106
C3	0.083	0.727	0.094	0.694
Cholesterol	0.061	0.798	0.007	0.977
Triglyceride	-0.409	0.073	-0.360	0.119
WBCs	0.198	0.403	0.300	0.198
HB	0.382	0.096	0.526	0.017
PLT	-0.105	0.658	-0.178	0.452
MCV	-0.086	0.719	0.033	0.889
MCH	-0.124	0.604	-0.002	0.994

Table (**9**) shows that PC.c has negative correlation with Albumin, TP, PT while PC. Negative correlations with albumin, Total Protein, PT and positive correlation with Albumin /Creatinine Ratio and HB were found.

Table (10): Correlation between PC.c and PC.a as regards age and other variables in relapse stage:

	PC.c		PC.a	
	R	**P value**	**R**	**P value**
Age	0.135	0.570	0.135	0.570
Albumin	-0.999	0.001	-0.999	0.001
Total Protein	-0.918	0.001	-0.918	0.001
Albumin/Creatinine Ratio	0.989	0.001	0.989	0.001
PT	0.074	0.758	0.074	0.758
PTT	0.074	0.758	0.074	0.758
Creatinine	0.108	0.650	0.108	0.650
C3	-0.314	0.177	-0.314	0.177
Cholesterol	0.941	0.001	0.941	0.001
Triglyceride	0.965	0.001	0.965	0.001
WBCs	0.011	0.962	0.011	0.962
HB	-0.176	0.457	-0.176	0.457
PLTs	0.045	0.850	0.045	0.850
MCV	-0.606	0.005	-0.606	0.005
MCH	-0.381	0.098	-0.381	0.098

This table shows that PC.c has negative correlation with Albumin, Total Protein and MCH and positive correlation with Albumin/creatinine Ratio, Cholesterol and Triglyceride while PC. Negative correlations with Albumin, Total Protein, MCV and positive correlation with Albumin/creatinine Ratio, Cholesterol, Triglyceride were found.

DISCUSSION

In this study, the hemoglobin in gm/dL was found to be decreased in cases of relapse & remission compared to the control & the mean value of Hb in remission was increased than that seen in relapse.

On the other hand, RBCs count/cmm was found to be significantly decreased in case of patients in relapse in contrast to non-significant decreased in case of remission. The MCV was found to be very highly significant decreased in cases of relapse & highly significantly decreased in cases of remission.

MCH was found to be significantly decreased in cases of relapse & remission while MCHC was found to be very highly significant decreased in cases of remission.

From the foregoing, it can be concluded that, the patients had anemia& the type of anemia was hypochromic microcytic anaemia. The microcytic hypochromic anaemia in nephrotic patients has different etiologies among which are, loss of transferrin in urine which is essential for iron metabolism & lead to iron deficiency anaemia not responsible to iron

therapy. This conclusion was in accordance with that reported by **Mictchett *et al.*[12]**. Also malabsorption of iron from the intestine as in chronic renal diseases including N.S. might be the cause of iron deficiency anaemia.

In contrast to what was seen in this study, normocytic normochromic anaemia might be reported in some case of nephrotic syndrome either due to haematuria or deterioration of kidney functions [13].

The present study showed leucocytosis especially in active state (relapse) mainly polymorph type. Regarding WBCs count, one could imagine to find leucocytosis as a response to hemorrhage (haematuria) & / or to combat infection as N.S. patients are susceptible to recurrent infections[14].

In the present study it was found that platelet count not significantly increased in nephrotic syndrome (P > 0.05) in both groups (remission & relapse).Increased platelet count coincided with what had been previously reported by **Andre *et al.*[15]** might explain the thrombotic risk which has been reported in some cases of nephrotic syndrome.

In the present study , it was found that , there is a decrease in prothrombin time (PT) & activated partial thromboplastin time (APTT) which were non significantly decreased in cases of remission (P = > 0.05). In relapse PT was found to be very highly significant decreased (P = < 0.001) while APTT was found to be highly significant decreased (P = < 0.01) as compared to the control group. These results were in contrast to what was described by **Ueda *et al.*[16]** who stated that, PT was normal while APTT was prolonged during relapse. Also, the results were in contrast to what was described by **Ruggeri *et al.*[11]** who showed that PT & APTT level were within the normal range.

The causes of short PT, APTT in nephrotic syndrome is multiple &complex. These tests practically comprise the whole coagulation protein cascade whether extrinsic (factor VII &tiss factors), intrinsic (factor XII, XI, IX & VIII) or the final common pathway (factor X, II, I & XIII). Regarding total protein (gm/dL) & serum albumin (gm/dL). Our results showed that, the both were very highly significantly decreased (P <0.001) in relapse & remission as compared to the control.

These results could be explained by the fact that, proteinuria , including albuminuria is the initial & single most diagnostic clinicolaboratory finding in patients with nephritic syndrome. The excess excretion of protein is due to the increased glomerular Alteration of protein resulting from the increased permeability of the glomerular basement membrane. Massive urinary loss of albumin is undoubtedly a major factor in hypoalbuminaemia, but decreased synthesis, increased catabolism or extrarenal losses are additional factors [17].

Regarding serum Triglyceride (gm/dL) & serum Cholesterol (gm/dL). Our results showed that, the both were very highly significant increase (P <0.001) in relapse & remission as compared to the control.

Regarding the position of P-C in nephrotic syndrome, P-C as mentioned before is synthesized in the liver to circulate as inactive zymogen. Thrombin is the physiological activator of P-C in the presence of an essential endothelial cell membrane cofactor, thrombomodulin. Activated P - C not only possesses anticoagulant properties but also stimulates the fibrinolytic activity in vivo through the inactivation of plasminogen activator inhibitor[18].

It was believed that P-C may be lost in the urine of patient with severe proteinuria & consequently the plasma level of P-C may be decreased, but in our study there was a very high significant increase in P-C, both in activity & concentration in relapse but in remission, its level decreased towards the normal level but still slightly increased & this increase is statistically non-significant (P >0.05).

As regards to our results & the previous reports that plasma P-C levels were much increased in nephrotic patients; two possibilities are suggested, the first one is that the increase of P-C in patients with nephrotic syndrome is probably related to an increase in the synthetic rate by the liver which is secondary to proteinuria as discussed before, the second possibility is that P-C carries a highly negative charge[19].

The present study showed that there was a negative correlation between P-C level in plasma & serum albumin level & a positive correlation between P-C level & severity of proteinuria. The massive loss of albumin in urine leads to sharp decrease in the level in plasma & subsequent increase capacity of liver to synthesize proteins including albumin, P-C & other some coagulation proteins.

As regard P-C activity , there was a very high significant increase in the activity in relapse as compared to the control group , but in remission , its level of activity decreased but still above to the normal level, which was statistically non significant . There was a negative correlation between P-C activity & serum albumin, serum protein. This may be attributed to more synthesis of proteins in the liver, including P-C, & absorption of vitamin K. which help the gamma carboxylation of glutamic acid of P–C.

P-C activity and concentration were found to be significantly increased in nephrotic syndrome when compared with the normal control group. This discrepancy reflects a dynamic balance between urinary losses & systemic production[15].

CONCLUSION AND RECOMMENDATIONS

From the foregoing we can concluded that, Increase level of P-C in N.S. denoted that it has no role in thromboembolic complications and Increase level of P-C in N.S may afforded some protection against the

thrombotic diathesis by counteracting hypercoagulability state.

It is recommended, to estimate P-C in nephrotic syndrome for discovery of relapse state after remission, follow up of treatment & assessment the prognosis of the condition.

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