Increased platelet count and aggregability due to urinary loss of PACAP in congenital nephrotic syndrome

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Introduction

Thrombotic complications occurring in up to 15% of patients represent a severe burden in congenital nephrotic syndrome (CNS). (1) The underlying mechanisms are multi-factorial and are mainly unraveled in regard of the venous thrombosis, while elevated blood platelet counts and platelet hyperaggregability increase the risk of arterial thrombosis. The pituitary adenylate cyclase-activating polypeptide (PACAP) is a highly conserved neuropeptide. (2) The role of PACAP as an inhibitor of megakaryocyte maturation and platelet function has recently been established. (3) PACAP interferes with the regulation of apoptosis in megakaryocytes, via stimulation of NFκB signaling. (4) Because PACAP in plasma is bound to ceruloplasmin (5), we assumed that urinary loss of ceruloplasmin in CNS might lead tot PACAP deficiency, leading to thrombocytosis and increased platelet reactivity.

The aim of this study was to investigate plasma PACAP levels in relation to blood platelet counts and aggregability in patients with CNS and to examine if addition of recombinant PACAP changes growth of hematopoietic stemcells and differentiation into megakaryocytes in CNS.

1. Singhal et al. Thrombosis research. 2006; 118(3): 397-407. 2. Vaudry et al. Pharmacological reviews. 2009; 61(3): 283-357. 3. Freson et al. Blood. 2008; 111(4): 1885-93. 4. Di Michele et al. Molecular & cellular proteomics. 2012; 11(1). 5. Tams et al. The Biochemical journal. 1999; 341(2): 271-6.

Patients

Four patients with CNS of the Finnish type, aged 0.5-19 months, were tested. Patient A and B are sisters. All patients had a typical neonatal presentation with excessive urinary protein losses, hypoalbuminemia, low plasma ceruloplasmin and elevated platelet counts (table 1). Patient A, B and C underwent a bilateral nephrectomy. In these patients we could perform tests during nephrotic stage before nephrectomy and in a non-nephrotic stage after nephrectomy.

Table1: Clinical characteristics of patients

	Patient A		Patient B		Patient C		Patient D		Reference values
Gender	F		F		M		M		/
Mutation in *NPHS1*	homozygous c.514-516del		homozygous c.514-516del		compound heterozygous c.896G>C and c.2479C>T		compound heterozygous c.710T>C and c.514-516del		/
Age at sampling (months)	19	33	0.5	20	1	13	3	/	/
Platelet count (*1000/µL)	581	367	494	259	591	232	461	/	150-450
Plasma albumin (g/L)	16.4	48.3	18.2	42.6	28.0	39.3	19.5	/	35.0-52.0
Serum ceruloplasmin (g/L)	0.08	0.43	0.08	0.26	0.11	0.32	/	/	0.22-0.58
Plasma creatinine (mg/dL)	0.29	/	0.37	/	0.39	/	0.24	/	0.26-0.42
Plasma PACAP (% of control plasma pool)	24.6	79.5	38.1	84.4	14.7	106.2	40.7	/	100.0
Urine proteins (g/L)	19.7	/	17.0	/	12.2	/	16.9	/	<0.2
Urine PACAP (% of control plasma pool)	110.4	/	107.5	/	69.5	/	41.2	/	3.0

Results

1. All patients in nephrotic stage had plasma PACAP deficiency (14-40%, p<0.001) and excessive urinary PACAP excretion (figure 1,2).

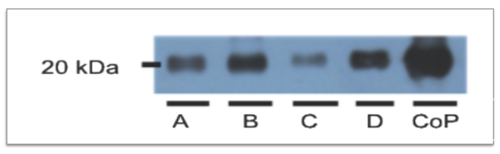

Figure 1: Immunoblot analysis of PACAP in plasma samples. A-D: CNS patients, CoP: control plasma pool.

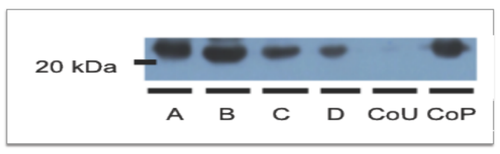

Figure 2: **Immunoblot analysis of PACAP in urine samples.** A-D: CNS patients, CoU: control urine sample, CoP: control plasma pool.

2. In patient A, B and C a bilateral nephrectomy was performed because of ongoing very severe nephrosis despite full treatment. In these three patients, we saw a significant increase in plasma albumin, plasma PACAP and serum ceruloplasmin and a significant decrease in platelet count after nephrectomy (figure 3). A strong correlation (R2 > 0.95) was found between platelet count and plasma PACAP levels (figure 3).

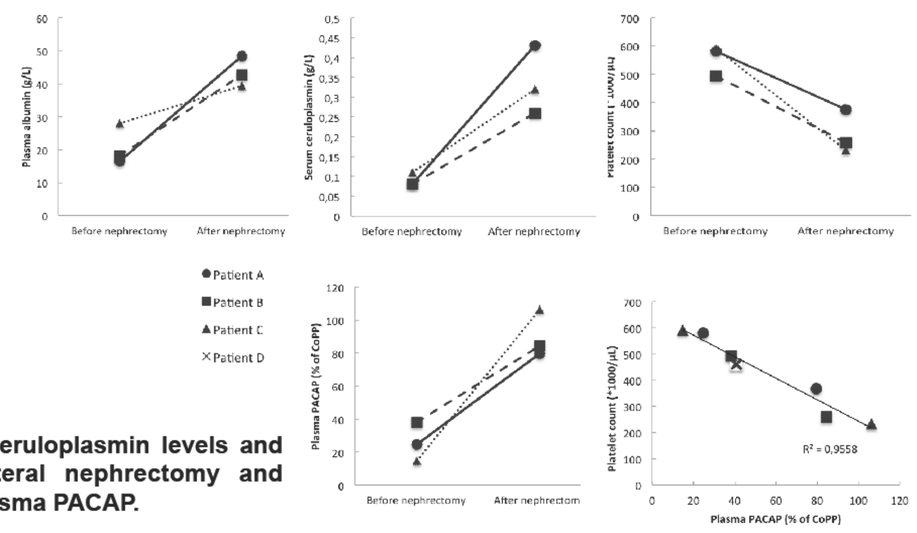

Figure 3: Plasma albumin, PACAP and ceruloplasmin levels and platelet counts before and after bilateral nephrectomy and correlation between platelet count and plasma PACAP.

3. In patient A, plasma PACAP levels and platelet counts were measured the day before and the days after bilateral nephrectomy. Plasma PACAP levels progressively rose within days after nephrectomy (figure 4) and blood platelet counts normalized (figure 5).

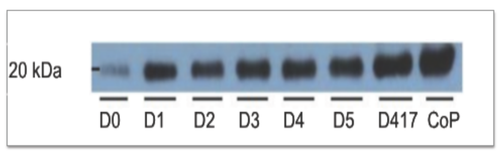

Figure 4: Immunoblot analysis of PACAP in plasma of patient A, daily during 5 days after bilateral nephrectomy and after 417 days.
D0-417: day after nephrectomy, CoP: control plasmapool.

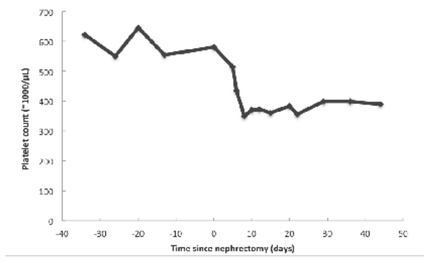

Figure 5: Blood platelet counts in plasma of patient A, at different time points from 1 month before to 1 month after bilateral nephrectomy.

4. Hematopoietic stemcells were isolated from peripheral blood in patient B and C and *in vitro* differentiated into colony forming unit (CFU) megakaryocytes. In one condition PACAP (1µM) was added to the culture dish on day 0, 4 and 8, while in the other condition no PACAP was added. There was a significantly lower amount of colonies after addition of PACAP in nephrotic stage (figure 6).

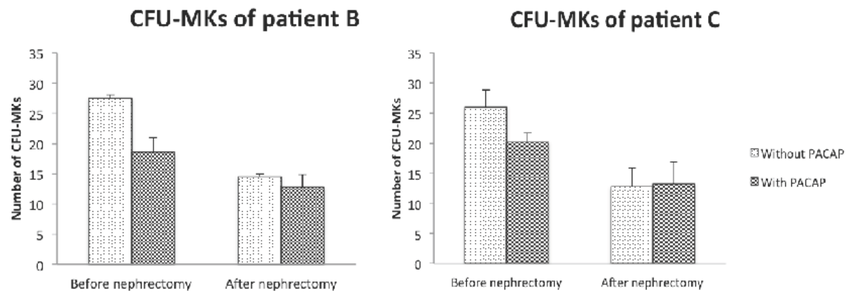

Figure 6: Amount of colonies of CFU megakaryocytes after 11 days of culture, with and without addition of PACAP.

5. In patient B and C platelet aggregation was tested before and after bilateral nephrectomy. In analogy to PACAP deficient mice, an increased platelet aggregation response to collagen was found during nephrotic stage, while platelets after bilateral nephrectomy showed normal reactivity towards collagen (figure 7).

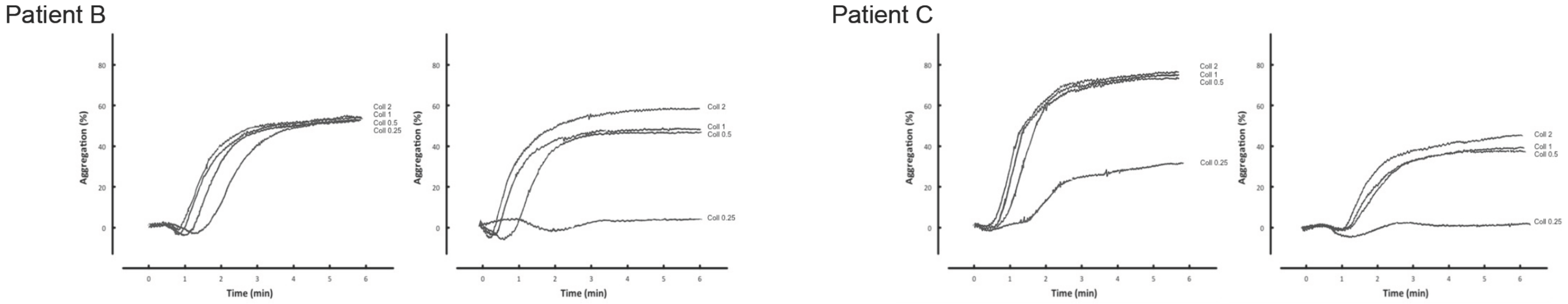

Figure 7: Platelet aggregation responses after addition of different collagen concentrations.

Conclusion

Our observations provide new insights on the mechanisms of arterial thrombosis in NS and is a proof-of-principle that PACAP deficiency exists in CNS. In analogy to mice, PACAP deficiency in CNS seems to play an important role in the thrombocytosis, by stimulating megakaryopoiesis, and in platelet hyperaggregability. When confirmed in larger studies, PACAP replacement or stimulation of PACAP receptors might become a valuable therapeutic option for prevention of arterial thrombosis in NS.

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