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ORIGINAL ARTICLES

Assessment of serum RANKL in Children with Nephrotic Syndrome: Its Relation to Glucocorticoids Treatment

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ABSTRACT

Nephrotic syndrome (NS) is considered as a common chronic disorder. Its prevalence in children is 15 times greater than adults. Receptor Activator of Nuclear Factor-Kappa B Ligand (RANKL) is one of the proteins in the tumor necrosis factor (TNF)/TNF receptor families required for the control of bone remodeling. The aim of this study was to assess the serum level of RANKL in children with idiopathic nephrotic syndrome (INS) in different stages of the disease (remission & relapse) and its relation to glucocorticoids treatment. The study comprised 40 children diagnosed as having INS. Twenty children were in relapse and the other 20 were in remission. Another 20 healthy children served as control group who were matching in age and sex involved in the study. All the studied children were screened for their anthropometric measurements (height, weight, Body mass index (BMI) for-age Z-score), clinical parameters and laboratory assessment; (serum RANKL, albumin, cholesterol, A/C ratio, calcium, phosphorus, ALP, and creatinine). Results of this work revealed that sRANKL concentration was significantly higher in the relapse group as compared to the control group (p=0.002). No similar difference was noted between remission and control. In addition the results showed a significant positive correlation between the duration of glucocorticoids (GCS) treatment and the concentration of sRANKL in both groups of NS patients. It was concluded from this study that treatment with GCS for a long time results in bone affection, so sRANKL can be used as a detector of bone affection and abnormality and accordingly early intervention and prevention of complications can take place.

Key words: Nephrotic syndrome, Glucocorticoids, sRANK

Introduction

Nephrotic syndrome is a clinical syndrome characterized by the tetrad of oedema, hypoproteinemia, proteinuria and hyperlipidemia Its prevalence in children is 15 times greater than in adults **(Guha *et al*., 2009)**.

Corticosteroids are the standard first-line of treatment in nephrotic children. Children are given high doses over a long period due to the chronic and recurrent nature of the disease. This increases the risk of osteoporosis (steroid induced osteoporosis) which in turn has long term implications (Gulati *et al*., 2009).

A set of proteins in the tumor necrosis factor (TNF)/TNF receptor families were reported to be required for the control of bone remodeling. These receptors, namely, Receptor Activator for Nuclear Factor Kappa B (RANK), osteoprotegerin (OPG), and the RANK ligand (RANKL), were identified as forming a critical molecular triad that controls bone remodeling (Hofbauer *et al*., 2000). The binding of RANKL to its receptor (RANK) induces differentiation, activation, and prevention of osteoclast apoptosis, leading to enhanced bone resorption and bone loss (Khosla, 2001).

GCS stimulate the differentiation and function of osteoclasts, which are cells derived from the monocyte/macrophage family, differentiating under the control of two cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL)(Chiodini *et al*., 1998).

GCS increase the expression of M-CSF and RANKL and decrease the expression of RANKL soluble decoy receptor-OPG produced by the osteoblasts (Hofbauer *et al*., 1999). Consequently, OPG blocks the binding between RANKL and RANK, thereby playing the role of the major inhibitor of osteoporosis.

The RANKL/OPG ratio seems to be the key mechanism in the pathogenesis of glucocorticoid-induced osteoporosis (GIOP) glucocorticoid-induced osteoporosis (Chiodini *et al*., 1998).

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Few clinical reports have been published on the role of serum RANKL and OPG levels in the diagnosis of glucocorticoid-induced osteoporosis. Oelzner *et al*., (2007) demonstrated that high serum levels of RANKL were associated with osteoporosis in patients with rheumatoid arthritis, but influences of age and gender must also be considered. Turk *et al*. (2009) found that free sRANKL and OPG showed a highly inverse relationship in patients with reduced bone density in the course of Crohn disease .

Recently Wasilewska *et al*., (2010) observed that INS children treated with GCS had an increased serum RANKL level, decreased serum osteoprotegerin (OPG) level, increased RANKL/OPG ratio. Their results also revealed a significant positive correlation between the cumulative dose of GCS and the serum RANKL. They also found that concurrent GCS treatment increased the serum RANKL level and the latter correlated negatively with the bone mineral density (BMD) (Bone mineral density) Z-score.

Materials and Methods

This study was conducted on 40 children who were recruited from the outpatient nephrology clinic of Children Hospital, Cairo University. These patients were diagnosed as having idiopathic nephrotic syndrome (INS) according to the definition of the International Society of Kidney Disease in Children (1981). They were 18 females and 22 males. Their ages ranged from 5 to 15 years with a mean of (8.5±3.6 years).

According to the clinical status and laboratory investigations we chose 20 patients in the relapse phase (group1). They were 11 females and 9 males. Their mean age was 8.1 ± 3.8 years. These patients showed either a deterioration in the level of albumin, cholesterol and albumin to creatinine ratio on follow up, or their Urinary protein excretion was >40 mg/m2/h; > 3+ by dipstick for 3 consecutive days (Bagga and Strivastava, 2005).

The other 20 patients were in the remission phase (group2). They were 7 females and 13 males. Their mean ages was 8.8 ± 3.6 years. These patients

showed either an improvement in the serum level of albumin, cholesterol and albumin to creatinine ratio on follow up or Urinary protein excretion was <4 mg/m2/h; nil or trace by dipstick on spot sample for 3 consecutive days (Bagga & Strivastava, 2005).

Inclusion criteria of patients were age <16 years, normal GFR (>90 ml/min /1.73 m2) ,and a history of systemic GCS therapy for NS for ≥ 1 month . The exclusion criteria were presence of clinical and laboratory findings of a systemic disease, and presence of congenital and secondary NS .

Another 20 healthy, age and sex-matched children who did not have any history of any illnesses or medications that might have affected their growth, or nutritional status served as control group (group 3). This group included 9 females and 11 males. The mean of their ages was 8.8 ± 3.7 years.

All the studied children were subjected to careful clinical history and physical examination. Height, weight measurement and body mass index (BMI) calculation were expressed as weight-for-age Z-score, height-for-age Z-score (HAZ), and BMI Z-score with reference to the WHO standards using Anthro plus Software of WHO (2009).

Blood samples were drawn from all patients and children in the control group to measure the concentration of serum RANKL, creatinine, albumin, cholesterol, calcium, phosphorus, and alkaline phosphatase .

The concentration of serum RANKL was measured using human sRANKL (TOTAL) ELISA (enzyme linked immune sorbent assay) with cat. No. RD193004200R according to the method of (Lam *et al*., 2001).The normal value of RANKL was 339.34 ± 42.3 pmol/l .

Statistical analysis:

The data were analyzed using statistical package for social sciences (SPSS) version 13.2, Echosoft corp, USA, 2003. Results were presented as mean ± SD, except where otherwise indicated. Comparisons between groups were done by the t test for unpaired observations and the Mann-Whitney test, as appropriate. Correlations between two variables were obtained by the Pearson linear regression analysis or by the Spearman rank correlation coefficient. P<0.05 was considered significant.

Ethical Consent was obtained from the parents of each child after explaining the aim of the study. 3

Results:

The anthropometric characteristics of the studied children with INS and control group are summarized in **table (1**). The age of both groups and the male to female ratio were similar. Significant higher weight Z-score (p=0.003), lower height Z score (p=0.01), and greater BMI Z-score (<0.001) were observed in nephrotic children as compared to control children. In nephrotic children we found a higher percentage of overweight (37.5%, n=15), obesity 45% (n=18) and short stature (57.5%).

Table (2) shows the disease characteristics of both groups of patients with INS. The duration of glucocorticoid treatment was significantly longer in patients with relapse (p=0.03).

Table 1: Anthropometric characteristics of nephrotic patients (INS) versus control.

INS n=40		Control	P value
		n=20	
age (years)	8.5±3.6	8.83±3.7	NS
M/F ratio		22/18	11/9
HAZ	-1.17±1.5	-0.22±0.41	0.01*
WAZ	1.5±1.12	0.32±0.51	0.003*
BMZ	2.3±1.6	0.6±0.4	0.001*
Overweight (BAZ=1:2)	N=15 (37.5%)	N=3 (15%)	0.00*
Obese (BAZ >2)	N=18 (45%)	N=0 (0%)	0.00*
Short (HAZ <-1)	N=23 (57.5%)	N=1 (5%)	0.00*

Table 2: Clinical characteristics of the relapse group and the remission group.

		Relapse (n=20) N %	Remission (n=20)N %	P value
Type of NS	SDNS	18 90%	15 75%	NS
SRNS			2 10%	5 25%
Pathology	MCNS	16 80%	17 85%	NS
non MCNS			4 20%	3 15%
No of relapse	Frequent¤	14 70%		
	infrequent	6 30%		
Glucocorticoid duration (ys) mean ±SD	3.975±2.302		2.725±1.175	0.03

¤ Frequent relapses: means two or more relapses in 6 months of initial response or 4 or more relapses in any 12 month period

The biochemical parameters of both groups of nephrotic children and control group are shown in **table (3).** The serum creatinine, alkaline phosphatase and phosphorus levels did not differ between either group of INS children and the control. The mean level of RANKL in the relapse group (G1) was significantly higher compared to that of the control group (p= 0.004), however no significant difference was observed between remission group(G2) and control group.

Table (4) shows that on comparing the biochemical parameters of the 2 groups of patients, the levels of Ca and RANKL were significantly higher in the relapse group (p=0.03 and 0.02 respectively). 4

No significant difference was observed between males and females of the three studied groups as regards the levels of s RANKL (**table 5**).

Table(3): Biochemical parameters of the two groups of patient and the control group .

	Relapse(G1)Mean± SD	Remission(G2) Mean± SD	Control(G3) Mean± SD	P
Ca (mg/dl)	9.3±1.6	8±2	10±1.2	0.001[a]
ph(mg/dl)	3.7±1.3	3.3±1.7	3.7±1.2	NS
ALP (U/I)	307.8±123	374.6±148.9	302±136.4	NS
Creatinine(mg/dl)	0.36±0.2	0.37±0.1	0.46±0.2	NS
RANKL(pmol/L)	439±176.6	320.25±132.8	291±99.3	0.002[b]

Table 4: Comparison between the relapse group and the remission group as regards the biochemical parameters.

	Relapse Mean± SD	Remission Mean± SD	t	p value
Ca (mg/dl)	9.3±1.6	8±2	2.19	0.037
ph(mg/dl)	3.7±1.3	3.3±1.7	0.84	NS
ALP (U/I)	307.8±123	374.6±148.9	-1.54	NS
Creatinine(mg/dl)	0.36±0.2	0.37±0.1	-0.3	NS
RANKL(pmol/L)	439±176.6	320.25±132.8	2.4	0.028

Table 5: Difference between males and females as regards the mean level of RANKL in the 3 studied groups.

	Male Mean ± SD (pmol/L)	Female Mean ± SD (pmol/L)	t	p
Relapse	417.4±173.2	456.6± 185.7	-0.48	NS
Remission	336.8± 118.4	289.6± 161.7	0.75	NS
Control	265.1± 112.1	332± 75.8	-1.29	NS

In our study there was a highly significant increase in the level of sRANKL in the remission group patients with minimal change nephritic syndrome (MCNS) compared to the non minimal change nephritic syndrome (non MCNS) patients (353.6 ± 113.1 versus 131.0 ± 45.0 p<0.01). No similar difference was detected in the relapse group .

We found that in the relapse group the mean level of RANKL was significantly higher in patients with frequent times of relapses(660.3 ± 62.4 pmol/L) compared to that in patients with infrequent times of relapses (344.1 ± 108.6 pmol/L) (p=0.001).

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The results of this study showed a significant positive correlation between level of RANKL and the duration of glucocorticoids treatment in both groups of patients **{figure 1, 2}.** We also found a significantly negative correlation between level of RANKL and age of control group **{figure 3}.** However, no similar correlation was found in nephrotic patients.

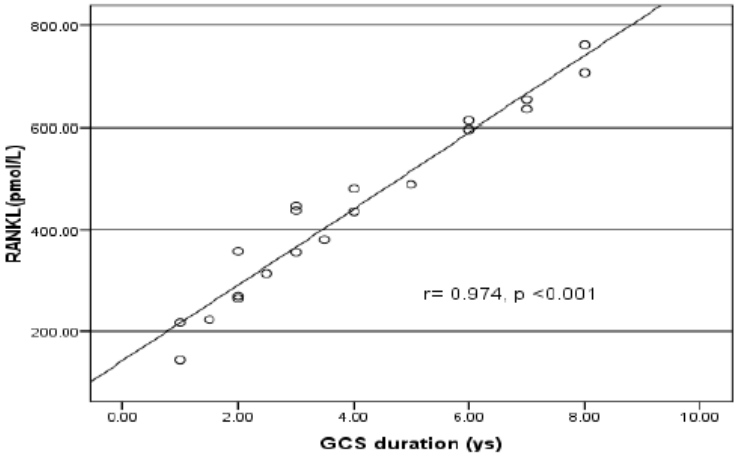

Fig. 1: Correlation between the duration of glucocorticoids (GCS) treatment and RANKL level in the relapse group.

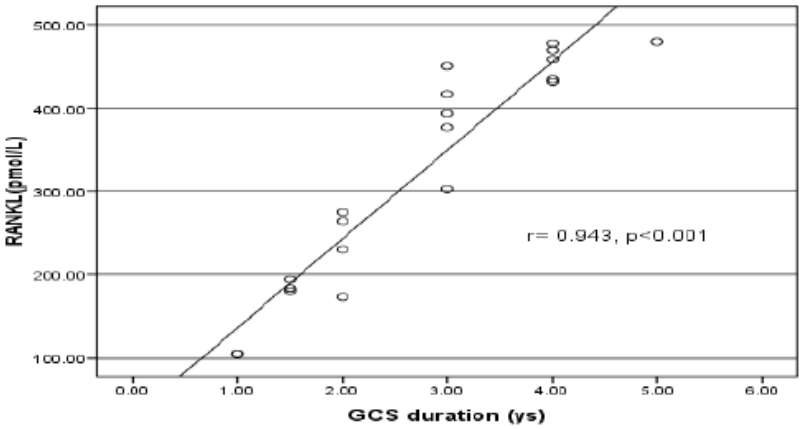

Fig. 2: Correlation between the duration of glucocorticoids (GCS) treatment and RANKL level in the remission group.

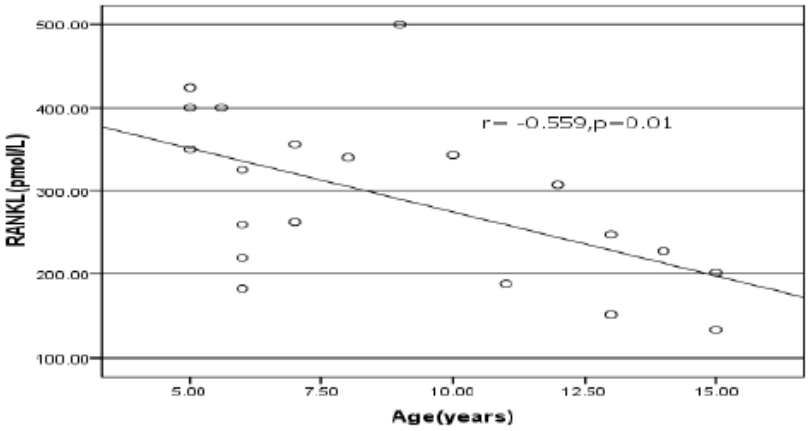

Fig. 3: Correlation between age and sRANKL in the Control group.

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Discussion:

In our study, the anthropometric parameters showed a significantly higher weight Z-score (p=0.003), lower height Z score (p=0.01), and a greater BMI Z-score (p=0.001) in the nephrotic children compared to those of control group. In addition, our data also showed that a greater percentage of nephrotic children were overweight and obese (37.5% , 45% respectively) compared to those of the control group (3% and 0% respectively) and 57.5% (n= 23) of nephrotic children were short stature. These results are in accordance with those reported in the study of Mary (2007),who revealed that subjects with nephrotic syndrome had significantly decreased height (p=.008), and increased BMI Z scores (p= .001) compared with controls. Almost similar percentage of obesity (38%) was detected among their studied children .

Moreover, our results are matching with those in the study of Wasilewska *et al*., (2010) who found significantly lower height Z-scores (p<0.05), greater BMI Z-scores (p=<0.01), and a greater prevalence of obesity and short stature in their patients with INS. These results are explained by the fact that prolonged glucocorticoids treatment affects growth and fat distribution.

Among our nephrotic patients, there were significant differences between males and females as regards weight Z score (1.64 ± 1.06 vs 0.55 ± 0.91 p= 0.001) and body mass index Z-score (2.91 ± 1.92 vs 1.67 ± 1.45 p= 0.03 respectively). In contrast to our results, the study of Foster *et al*., (2004), revealed absence of gender difference in the anthropometric results of their nephrotic patients .

Our results showed that the serum phosphorus concentration was normal in both the relapse and the remission group. This result was reported by many studies who revealed that serum phosphorus concentration are rarely elevated in nephrotic syndrome (Korkor *et al*., 1983 and Freundlich *et al*., 1985).

The calcium level was significantly lower in the remission group as compared to that of the control group (8±2 and 10±1.2mg/dl respectively), (p=0.001). Disturbances of mineral metabolism that potentially affect bone integrity have been recognized in patients with NS before the development of renal insufficiency and include hypocalcemia and reduced intestinal calcium absorption (Lettgen *et al*., 1994).

In our work the mean level of RANKL was significantly higher in the relapse group of NS as compared to that of the control group (p= .002) . On the other hand, no similar difference was observed between remission group and control group. In addition, the difference in the level of RANKL between relapse group and remission group was statistically significant (p= 0.02). These results suggest that changes in RANKL expression in children with NS are transient and reversible . It was stated that steroid-sensitive nephrotic syndrome in relapses are associated with transient increases in cytokines, and these abnormalities promptly resolve with glucocorticiod therapy and disease remission (Daniel *et al*., 1997). However, the study of Freundlich *et al*., (2005), reported that changes in osteoblastic RANKL expression were not influenced by the concurrent administration of steroid.

On the other side, the study of Faienza *et al*., (2009), showed increased concentration of RANKL and decreased concentration of OPG in patients with a 21 hydroxylase deficiency who were treated with GCS compared with control. Furthermore, Wasilewska *et al*., (2010), reported that sRANKL concentration was significantly higher in their patients with NS compared to that of control group.

The results of this study showed a significant positive correlation between the duration of glucocorticoids treatment and the level of sRANKL in both groups of patients (r = 0.9, P = 0.0001and r=0.9, p=0.0001). This correlation was previously reported in the study of Wasilewska *et al*., (2010) who found a positive correlation between cumulative dose of glucocorticoids and the concentration of sRANKL (r=0.33, p<0.05) . They also revealed that serum RANKL concentration was significantly higher in the group of NS children treated with high dose of GCS in comparison to the group of patients who had minimal exposure to GCS.

Since our subject is still not widely dealt with especially concerning human beings, therefore we had to refer to some animal studies and other in vitro studies in order to widen our scope until further studies are done and reported. Accordingly, we could say that some of our study results are matching with the animal study results done by Lacey *et al*., (2007) who showed that when soluble (sRANKL) was administered to mice, an increase in osteoclast formation and activation occurred and led to osteoporosis and hypocalcaemia. RANKL knockout mice on other hand revealed an increase in bone mass (osteopetrosis) and impaired tooth eruption because of the lack of mature osteoclasts (kong *et al*., 2009). Glucocorticoids 7 have been shown to influence RANKL production in vitro as shown by Hofbauer *et al*., (2003) who demonstrated an increase in RANKL production by osteoblast-like cells in response to dexamethasone

Our results showed that no significant difference in serum RANKL between males and females in either nephrotic patients or control. We also found that level of sRANKL was negatively correlated with age in healthy children (r=0.5 p=0.01), but not in nephrotic patients.

In accordance to our results the study of Buzi *et al*., (2004), revealed that there was a decline in RANKL levels with increase age in normal healthy children. However, Wasilewska *et al*., (2009), showed that the level of sRANKL was 3 times higher in males than in females and almost 3 times higher in older than younger children.

Furthermore, Wasilewska *et al*., (2010), stated that the incidence of increased sRANKL was not significantly associated with the age >10 years, height < 3 centil and over weight >90 centil.

In our study the highly significant increase in the level of sRANKL in the remission group patients with minimal change nephrotic syndrome (MCNS) compared to the non minimal change nephrotic syndrome (Non MCNS) patients is explained by the fact that MCNS patients in the remission phase are always steroid dependent and receive high doses of steroids for a long period of time to maintain remission and steroids cause an increase in RANKL level.

In contrast to our study, two studies have showed that the different NF-κB level in nonMCNS was higher and accordingly this provides a theoretical basis for early administration of NF-κB inhibitors as a sound approval for the prevention and treatment of primary nephrotic syndrome. They also stated that activated NF-κB exerts an inhibitory effect on glucocorticoids receptors and accordingly the higher activities of NF-κB in nonMCNS may be the cause of the poor response to steroids therapy (Cao *et al*., 2001 and Hong-yang *et al*., 2005) .

The mean level of RANKL is significantly higher in patients with frequent times of relapses compared to that in patients with infrequent times of relapses (660.3 ± 62.4 versus 344.1 ± 108.6 pmol/L) respectively (p=0.0001). This is due to the fact that patients with frequent times of relapses receive high doses of steroids for a long time and steroids cause an increase in sRANKL level.

The serum RANKL level was significantly higher in steroid dependent patients compared to its level in steroid resistant patients (412.6±155.04 versus 224±125.2 pmol/L) respectively (p=0.005). This is due to the longer course and the higher dose of steroids in steroid dependent patients.

In conclusion, children with INS treated with GCS had an increased serum RANKL level. There was a strong positive correlation between the duration of steroids treatment and the increase in serum RANKL level. Our study also confirmed the fact that treatment with glucocorticoids for a long period of time in some diseases as nephrotic syndrome results in different side effects as obesity, short stature and bone affection.

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