Association between serum soluble tumor necrosis factor- α receptors and early childhood obesity

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Abstract. This study aimed to characterize the inflammatory cytokine profiles and further validate the significantly different cytokines in the serum obtained from obese children aged 36–48 months. Four obese children and four lean controls were randomly selected for inflammatory cytokine array assay, in which two cytokines [soluble tumor necrosis factor- α receptors (sTNFRs) 1 and 2] were found to be significantly different. Both cytokines (sTNFR1 and sTNFR2) were then further validated through enzyme-linked immunosorbent assay (ELISA) in 61 obese children and 52 lean children. ELISA results revealed that serum sTNFR1 level in obese children significantly increased ($p = 0.003$), whereas sTNFR2 did not change significantly ($p = 0.069$). Stratified analysis by gender showed that only obese girls presented increased sTNFR1 ($p = 0.005$) and sTNFR2 ($p = 0.049$) levels. We conclude that serum sTNFR1 is elevated in early childhood obesity. Moreover, serum sTNFR1 and sTNFR2 are associated with obese girls but not obese boys, thereby suggesting that serum sTNFRs in early childhood obesity may be sex related.

Key words: Child obesity, Early childhood, sTNFR, Inflammatory cytokine

OBESITY and childhood obesity are major public health problems worldwide. Increasing evidence indicates that obesity is causally linked to a chronic low-grade inflammatory state [1, 2]. Obesity in childhood increases the risk of obesity in adulthood, which in turn, is associated with the development of obesity-related disorders.

The chronic low-grade inflammatory state is characterized by elevated circulating levels of inflammatory cytokines and acute-phase proteins [3, 4]. A review [5] summarized that adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines, such as adiponectin and interleukin (IL)-10. In contrast, obese adipose tissue mainly releases pro-inflammatory cytokines, such as tumor necrosis factor (TNF)- α , IL-6, and leptin. Some circulating cytokines, including TNF- α , high-sensitive C-reactive protein (CRP), IL-6 and adiponectin, have been correlated with human obesity [6-8]. Most of the knowledge on obesity-induced inflammation comes from studies in

adults, adolescents, and older children. Only a few studies, such as those of Bocca *et al.* and Skinner *et al.*, focused on very young children, and even then, they only examined a few markers of inflammation [6, 9]. Early childhood obesity provides an excellent opportunity to study inflammatory changes without confounding lifestyle habits (*e.g.*, smoking) and co-existing inflammatory conditions (*e.g.*, cardiovascular disease or arthritis) [7]. Therefore, the data on relationship of obesity with very young obese children need to be enriched urgently.

In the present study, we characterize the inflammatory cytokine profiles in serum of obese children at 36–48 months, using a Quantibody® Human Inflammation Array. To the best of our knowledge, no study has comprehensively explored inflammatory cytokines profiles in preschool children, especially children distributed at a narrowed age range (*e.g.*, 3-years-old). The inflammatory array has similar detection sensitivity as traditional enzyme-linked immunosorbent assay (ELISA), and allows the quantitative detection of 40 different inflammation-related cytokines simultaneously. Hence, this approach is used to identify novel inflammatory mediators associated with early childhood obesity.

Materials and Methods

Study population

A total of 121 children aged 36–48 months, who participated in a physical check for kindergarten enrollment, were recruited in the study. Children with an underlying hormone deficiencies, genetic disorders, inflammatory conditions, or occurrences of recent acute infections were excluded. Among the participants, 4 obese children and 4 lean controls were randomly chosen for array analysis in the discovery set. In the validation set, the study cohort consisted of 113 other subjects, including 61 obese children [body mass index (BMI) > 18.5 and < 26 kg/m²] and 52 lean children (BMI > 13.5 and < 15 kg/m²). Obesity classification criteria refer to BMI₂₈ of Chinese children aged 3 years [10]. The study was approved by the Institutional Ethics Committee of the Capital Institute of Pediatrics, Beijing, PR, China (SHERLLM 2012015).

Sample collection

Fasting blood samples were taken from the antecubital vein, and then centrifuged at room temperature for 10 min at 3,000 × g. Aliquots of serum were stored at -80 °C for subsequent use.

Human inflammation array

The collected serum was analyzed using the commercial arrays, Quantibody® Human Inflammation Array-3 (RayBiotech, Inc., USA), in accordance with the manufacturer's protocol. The multiplexed technology allowed simultaneous detection of 40 different cytokines.

ELISA

Levels of soluble TNF- α receptors (*i.e.*, sTNFR1 and sTNFR2) were measured by ELISA according to the manufacturer's instructions (Catalog Nos. DRT100, sTNFR1; DRT200, sTNFR2; R&D Systems, Inc., USA).

Statistical analysis

In the inflammation array assay, the results were expressed as means $\pm$ standard deviation (SD). The difference for every cytokine between obese and lean children was compared using student's *t*-test. In ELISA, data were expressed as median (25th–75th percentile), and both groups (obese *vs.* lean) were compared by Mann-Whitney U tests for being non-normally distributed. The differences in age and BMI between obese and lean children were compared using student's *t*-test. Difference in terms of gender was analyzed using Chi-square test. Stratified analysis was performed according to gender difference between two groups (obese *vs.* lean). All analyses were performed using SPSS 13.0 (Chicago, IL, USA), and statistical significances were denoted by *p* < 0.05.

Results

Basic characteristics of studied children

In the validation set, the basic characteristics of the recruited children are documented in Table 1. There exist statistically significant differences in gender (*p* = 0.008) and BMI (*p* < 0.001), but no statistically significant difference in age between the two groups (obese *vs.* lean). In the discovery set, the differences in terms of age, gender, and BMI between both groups were not compared for a small sample size (Supplementary Table 1).

Inflammation array analysis

The differences among the 40 cytokines were compared between obese and lean children. There were no significant differences in most of the measured parameters between the two study groups, except in serum sTNFR1 (*p* < 0.001) and sTNFR2 (*p* = 0.048). The results are shown in Supplementary Table 1 and Fig. 1.

Table 1 Characteristics of the participants in the validation set

	Lean (N = 52)	Obese (N = 61)	<i>p</i>
Age (month), mean $\pm$ SD	39.9 $\pm$ 3.1	39.9 $\pm$ 3.0	0.978 ^a
Gender (male/female)	16/36	34/27	0.008 ^b
BMI (kg/m ²), mean $\pm$ SD	14.1 $\pm$ 0.6	21.2 $\pm$ 2.2	< 0.001 ^a
sTNFR1, pg/mL (P ₂₅ –P ₇₅)	815.7 (703.7–916.3)	911.5 (773.0–1025.5)	0.003 ^c
sTNFR2, pg/mL (P ₂₅ –P ₇₅)	2,166.9 (1,891.2–2,534.8)	2,257.2 (2,087.2–2,731.6)	0.069 ^c

P₂₅, 25th percentile; P₇₅, 75th percentile; BMI, body mass index; sTNFR, soluble tumor necrosis factor - α receptor.

^a Student *t* test, ^b Chi-square test, ^c Mann-Whitney test

Fig. 1 Comparison of 40 inflammation-related cytokines between obese (Nos. 5–8) and lean (Nos. 1–4) children. Note: Color changes from green to red response the changes of cytokines levels from low to high in the samples.

Validation of cytokines by ELISA

The serum levels of sTNFR1 and sTNFR2 were further confirmed by ELISA for the respective differences found in the array analysis. The results reveal that obese children have significantly higher sTNFR1 levels than lean children ($p = 0.003$; Table 1; Fig. 2A). However, the level of sTNFR2 is not significantly changed between the two groups ($p = 0.069$; Table 1). Stratified analysis by gender shows that obese girls have higher sTNFR1 and sTNFR2 compared with lean girls ($p = 0.005$, $p = 0.049$, respectively; Table 2, Fig. 2B–2C). However, there is no significant difference in serum levels of sTNFR1 and sTNFR2 between obese and lean boys.

Fig. 2 Higher sTNFR1 (A) serum levels in children with obesity compared with controls. Girls with obesity presented higher sTNFR1 (B) and sTNFR2 (C) serum levels in comparison with lean girls using stratified analysis by gender. Note: The figure shows median and interquartile ranges. * $p < 0.05$, Mann-Whitney U test. sTNFR, soluble tumor necrosis factor- α receptor.

Table 2 Stratified analysis by gender

	Girls (N = 63)			Boys (N = 50)		
	Lean (N = 36)	Obese (N = 27)	<i>P</i>	Lean (N = 16)	Obese (N = 34)	<i>P</i>
Age (month), mean $\pm$ SD	40.1 $\pm$ 2.9	39.8 $\pm$ 2.9	0.721 ^a	39.4 $\pm$ 3.6	39.9 $\pm$ 3.1	0.634 ^a
BMI (kg/m ²), mean $\pm$ SD	14.1 $\pm$ 0.5	21.0 $\pm$ 2.1	< 0.001 ^a	14.0 $\pm$ 0.8	21.3 $\pm$ 2.2	< 0.001 ^a
sTNFR1, pg/mL (P ₂₅ –P ₇₅)	827.6 (718.0–916.3)	918.7 (842.8–1,113.7)	0.005 ^b	745.3 (668.9–926.1)	898.7 (753.7–975.3)	0.082 ^b
sTNFR2, pg/mL (P ₂₅ –P ₇₅)	2,136.0 (1,978.0–2,516.3)	2,353.6 (2,086.5–2,848.2)	0.049 ^b	2,285.4 (1,820.6–2,545.2)	2,212.7 (2,081.7–2,639.6)	0.603 ^b

P₂₅, 25th percentile; P₇₅, 75th percentile; BMI, body mass index; sTNFR, soluble tumor necrosis factor α receptor.

^a Student *t* test, ^b Mann-Whitney test

Discussion

TNF- α is the prototype of pro-inflammatory cytokine primarily produced by different cellular sources, including macrophages and lymphocytes. TNF- α exerts its effects through membrane-bound TNF receptors (TNFR) 1 and 2 [11]. TNFR1 and TNFR2 both contain four cysteine-rich repeats in their extracellular domains, forming elongated shapes that interact with the lateral grooves of the TNF- α trimer formed between two of its three protomers [12]. Soluble forms of the receptors (*i.e.*, sTNFR1, sTNFR2) also exist in blood and can bind to TNF- α [13].

In the present study, we validated a multiplex cytokine assay for the detection of human inflammatory cytokines, and identified two novel cytokines (*i.e.*, sTNFR1 and sTNFR2) in obese children aged 36–48 months. To the best of our knowledge, this is the first study to describe the association between sTNFRs and preschool childhood obesity. Some previous studies on the relationship between sTNFRs and human obesity have been performed, although their subjects are mostly adults, adolescents and older children. Some of these studies found that circulating sTNFR1 levels significantly increased in overweight/obese subjects, compared with their controls [14–18]. Meanwhile, other studies found significantly decreased circulating levels of sTNFR1 in obese subjects after performing weight loss intervention [19–21]. The abovementioned findings are in accordance with our findings, that is, obese children have a significant elevation in the serum levels of sTNFR1 compared with lean children. However, several other researchers [22–24] observed no difference of circulating sTNFR1 in the two groups (obese *vs.* lean). An increased level but no significant difference of serum sTNFR2 was observed in obese children compared with controls. The previous studies on the association between sTNFR2 and obesity also presented conflicting findings. Some studies reported that

the levels of circulating sTNFR2 are significantly elevated in obese children, adolescents and adults [15, 17, 18, 20–22], whereas others found inconsistent results, *i.e.*, sTNFR2 is not related to obesity [19, 23–26]. The inconsistent clinical findings of sTNFRs may attributed to the obesity phenotype [27, 28], degree of obesity [26, 27], gender [7, 17, 29], age [7], genetic and environmental factors [28], etc. The present study focused on the subjects distributed at a subdivided age (36–48 months) to demonstrate a decrease in age- and environment-related interference and provide reliable results.

Moreover, the significant elevation of serum sTNFR1 levels was only observed in obese girls based on the stratified analysis by gender. Interestingly, a marginally significant difference of sTNFR2 appeared between obese girls and lean girls, although serum sTNFR2 was not significantly correlated with obesity in all of the children. This finding indicates that the circulating inflammation cytokine response exhibits sexual dimorphism in obese children. Some previous studies have explored the sexual differences of circulating sTNFRs with human obesity [17, 28, 29]. In addition, other inflammatory markers, such as IL-10, TNF- α , IL-6, IL-8, and CRP, exhibit sexual dimorphism [30–32]. The mechanisms underlying these sexual dimorphisms may be multifactorial, such as the release of different hormones. Sex hormones have important interactions with the immune system and modulate the inflammatory response [30]. Sex hormone levels are relatively lower in childhood compared with other stages. However, the estradiol level in girls is apparently higher than the testosterone level in boys among children aged 3–4 years [33]. Estrogens may exhibit diverse effects on inflammation and immune response [32]. The results of the present study suggest that obese girls may be more sensitive to obesity-related inflammatory response than obese boys. Pamir *et al.* [34] demonstrated that TNFRs play a physiological role to limit body weight and adiposity by mod-

estly increasing the metabolic rate and fatty acid oxidation. sTNFRs at low concentrations can stabilize TNF- α activity by gradually binding/releasing this cytokine. However, high amounts of these receptors antagonize the biological effects of TNF- α . sTNFR1 and sTNFR2 are released in response to changing levels of TNF- α [35]. In the present study, cytokine array analysis revealed no significant difference ($p = 0.2$) in serum TNF- α between both groups (obese vs. lean). In consideration that further analysis to validate the serum level of TNF- α by ELISA was not performed, the associations of TNF- α with obesity in children, as well as with the sTNFR1 and sTNFR2 levels, were not assessed in the present study.

Moreover, some other studies have investigated the associations between circulating sTNFRs and other diseases, such as insulin resistance [17, 24, 36], metabolic syndrome [37] and type 2 diabetes [38], in patients with or without obesity. In the present study, preschool children aged 36–48 months were recruited to explore the association between serum inflammatory cytokines and obesity. The involvement of preschool children provides an excellent opportunity to study inflammatory changes during the early stages of obesity, without the confounding effects of lifestyle habits (e.g., smoking and co-existing inflammatory conditions). Furthermore, the interference factors, *i.e.*, age

and gender, are reduced by narrowing the age range and conducting stratified analyses, respectively.

However, several limitations of the present study should be considered. First, the sample size should be enlarged because of the marginal significance ($p = 0.048$) of sTNFR2 and the decreased power in the stratified analysis. Second, only the two cytokines (*i.e.*, sTNFR1, sTNFR2) were validated in this study for $p < 0.05$. Those cytokines with larger p values (e.g., $0.05 < p < 0.1$) should be further validated using ELISA.

Altogether, the present results demonstrate that serum sTNFR1 is elevated in early childhood obesity. Moreover, serum sTNFR1 and sTNFR2 are associated with obesity in girls but not in boys, thereby suggesting that serum sTNFRs in early childhood obesity may be sex related.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

Supplementary Table 1 The characteristics of participants and the results of inflammatory array including 40 cytokines in the discovery set

	Lean (N = 4)	Obese (N = 4)	P^a	Fold change ^b
Age (month), mean $\pm$ SD	38.7 $\pm$ 2.5	39.3 $\pm$ 2.7	–	–
Gender (male/female)	2/2	2/2	–	–
BMI (kg/m ²), mean $\pm$ SD	14.0 $\pm$ 0.5	23.2 $\pm$ 1.5	–	–
BLC	267.1 $\pm$ 213.9	332.3 $\pm$ 280.0	0.362	1.2
Eotaxin	70.7 $\pm$ 44.3	103.6 $\pm$ 70.0	0.229	1.5
Eotaxin-2	1,253.0 $\pm$ 730.5	1,969.0 $\pm$ 725.6	0.107	1.6
G-CSF	53.6 $\pm$ 23.7	56.4 $\pm$ 27.2	0.440	1.1
GM-CSF	323.3 $\pm$ 42.8	341.9 $\pm$ 38.6	0.271	1.1
I-309	55.8 $\pm$ 33.3	63.9 $\pm$ 38.0	0.379	1.1
ICAM-1	532.2 $\pm$ 156.7	635.6 $\pm$ 319.5	0.291	1.2
IFN γ	275.5 $\pm$ 24.0	291.1 $\pm$ 48.4	0.292	1.1
IL-10	362.2 $\pm$ 80.4	538.9 $\pm$ 286.0	0.140	1.5
IL-11	51.6 $\pm$ 14.2	44.2 $\pm$ 7.3	0.195	0.9
IL-12p40	29.6 $\pm$ 10.6	32.2 $\pm$ 15.6	0.397	1.1
IL-12p70	71.8 $\pm$ 22.3	61.8 $\pm$ 9.1	0.218	0.9
IL-13	165.7 $\pm$ 51.2	157.2 $\pm$ 38.6	0.400	0.9
IL-15	389.8 $\pm$ 247	340.5 $\pm$ 163.2	0.375	0.9
IL-16	313.4 $\pm$ 423.0	288.7 $\pm$ 249.4	0.462	0.9
IL-17	51.0 $\pm$ 9.1	43.5 $\pm$ 1.5	0.077	0.9

Supplementary Table 1 Cont.

	Lean (N = 4)	Obese (N = 4)	<i>P</i> ^a	Fold change ^b
IL-1 α	122.4 $\pm$ 24.8	119.2 $\pm$ 24.5	0.429	1.0
IL-1 β	13.8 $\pm$ 5.3	14.4 $\pm$ 9.9	0.456	1.0
IL-1ra	287.4 $\pm$ 18.2	268.0 $\pm$ 97.0	0.354	0.9
IL-2	137.1 $\pm$ 13.9	151.4 $\pm$ 31.2	0.217	1.1
IL-4	63.7 $\pm$ 39.8	53.3 $\pm$ 7.9	0.313	0.8
IL-5	132.4 $\pm$ 32.9	119.1 $\pm$ 4.6	0.228	0.9
IL-6	331 $\pm$ 39.5	376.4 $\pm$ 62.0	0.131	1.1
IL-6sR	7,971.7 $\pm$ 225.5	8,332.1 $\pm$ 327.5	0.060	1.0
IL-7	565.7 $\pm$ 252.6	461.6 $\pm$ 172.5	0.261	0.8
IL-8	357.9 $\pm$ 135.3	468.1 $\pm$ 258.4	0.239	1.3
MCP-1	1,391.0 $\pm$ 373.5	1,389.3 $\pm$ 252.5	0.497	1.0
MCSF	49.3 $\pm$ 65.6	23.9 $\pm$ 25.4	0.249	0.5
MIG	317.7 $\pm$ 227.2	270.1 $\pm$ 159.2	0.372	0.9
MIP-1 α	1,514.0 $\pm$ 621.0	1,668.9 $\pm$ 602.6	0.366	1.1
MIP-1 β	1,643.3 $\pm$ 640.7	1,824.1 $\pm$ 1,115.5	0.394	1.1
MIP-1 δ	374.8 $\pm$ 174.5	611.5 $\pm$ 277.2	0.099	1.6
PDGF-BB	14,122.4 $\pm$ 1,229.3	11,999.6 $\pm$ 3,179.9	0.130	0.8
RANTES	11,314.9 $\pm$ 782.1	9,537.2 $\pm$ 2,685.2	0.125	0.8
TIMP-1	3,853.9 $\pm$ 917.3	2,979.2 $\pm$ 702.0	0.090	0.8
TIMP-2	919.7 $\pm$ 128.6	953.9 $\pm$ 194.7	0.390	1.0
TNF sRI	58.2 $\pm$ 12.6	123.0 $\pm$ 14.0	0.000	2.1
TNF sRII	582.9 $\pm$ 86.0	807.0 $\pm$ 209.9	0.048	1.4
TNF α	338.1 $\pm$ 50.8	340.8 $\pm$ 57.0	0.473	1.0
TNF β	294.0 $\pm$ 42.0	288.5 $\pm$ 48.8	0.434	1.0

BMI, body mass index; 40 cytokines, refer to Quantibody® Human Inflammation Array-3 (RayBiotech, Inc., USA).

^a *p* value for student's *t* test between two groups (obese vs. lean), ^b The ratio of average level of cytokines between two groups (obese vs. lean)

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