The Impact of Childhood Obesity on Inflammation, Innate Immune Cell Frequency, and Metabolic MicroRNA Expression

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Background: Obesity is characterized by chronic inflammation, immune dysregulation, and alteration of gene expression, associated with type 2 diabetes mellitus and cardiovascular disease. The degree to which these changes occur in childhood obesity is not fully defined.

Aims and Methods: The aim was to investigate the effect of childhood obesity on immune cell frequency, macrophage activation, cytokine production, and specific regulators of metabolic gene expression. Profiling was performed on peripheral blood from 29 obese and 20 nonobese children using real-time PCR, ELISA, and flow cytometry.

Results: Fasting glucose was similar in both groups, but there was a higher degree of insulin resistance in obese subjects (homeostasis model of assessment for insulin resistance, 4.8 vs 0.84; $P < .001$). Soluble CD163, a marker of macrophage polarization to a proinflammatory profile, was elevated in the obese compared to nonobese children (135 vs 105 ng/mL; $P = .03$). Invariant natural killer T cells were reduced in the obese children (CD3 T cells, 0.31 vs 0.53%; $P = .001$). Cytokine profiling revealed significantly elevated TNF-α (6.7 vs 5.1 pg/mL; $P = .01$) and leptin (1186 vs 432 pg/mL; $P < .001$) and reduced adiponectin (884 vs 1321 pg/mL; $P = .001$) in obese compared to nonobese children. Stimulation of peripheral blood mononuclear cells from obese children resulted in higher levels of IL-1β (2100 vs 1500 pg/mL; $P = .018$). There was a 4-fold increase in expression of microRNA33a ($P = .001$) and a 3-fold increase in microRNA33b ($P = .017$) in obese children.

Conclusion: Childhood obesity is associated with changes in immune cell frequency, inflammatory environment, and regulation of metabolic gene expression. These changes have been causally linked to the onset of metabolic disease in adulthood and suggest the future trajectory of obese children to the development of type 2 diabetes mellitus and premature cardiovascular disease. (*J Clin Endocrinol Metab* 99: E474–E478, 2014)

Obesity is associated with the development of serious metabolic comorbidities, including premature cardiovascular disease and type 2 diabetes mellitus (T2DM).

The impact of weight on the immune and metabolic systems is now considered to be a dynamic bidirectional process. Immune system alterations in adult obesity are well described, but the impact of childhood obesity on immune status has been less well studied and is the focus of this work.

Inflammation underpins many obesity-related comorbidities. Proinflammatory cytokines are overexpressed in adult obesity and are linked to the metabolic syndrome (1). Similar proinflammatory profiles have been described in

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received September 18, 2013. Accepted December 6, 2013.
First Published Online December 18, 2013

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Abbreviations: BMI, body mass index; LPS, lipopolysaccharide; miR, microRNA; PBMC, peripheral blood mononuclear cell; sCD163, soluble CD163; T2DM, type 2 diabetes mellitus.

children, with reports of elevated C-reactive protein in obese children as young as 3 years (2–4). Inflammatory parameters such as IL-6, IL-8, IFN-γ, TNF-α, monocyte chemoattractant protein-1, and activated monocyte subsets have been noted to be elevated, and the anti-inflammatory adipokine, adiponectin, was reduced in obese compared to normal-weight children (4–7).

Macrophages play a distinct role in obesity-induced insulin resistance and are major contributors to adipose tissue inflammation (8). In healthy subjects, macrophages are regulatory M2 cells producing the anti-inflammatory cytokine IL-10. In obesity, macrophages are polarized to the M1 inflammatory phenotype, producing the proinflammatory cytokine IL-1β. As macrophages become proinflammatory, removal of the haptoglobin-hemoglobin receptor CD163 becomes up-regulated and is measurable as soluble CD163 (sCD163). sCD163 is strongly associated with insulin resistance independently of inflammatory parameters such as TNF-α (9). We recently identified a role for iNKT cells in the regulation of weight and metabolism in adult obesity (10). Depletion of iNKT cells in obese mice is associated with proliferation of M1 adipose macrophages (10).

This network of adiposity, immune cell dysregulation, and inflammation is interconnected with fatty acid metabolism and insulin signaling. We chose to study three critical gene regulators upstream from these metabolic pathways. MicroRNAs (miRs) are small, noncoding RNAs that modify post-transcriptional target gene expression and are strongly associated with metabolic disease in murine models and adult humans (11). MiR-33a and MiR-33b are expressed in multiple tissues including macrophages and target sterol-regulatory element binding protein, which regulates lipid and cholesterol homeostasis (12). MiR-107 regulates insulin sensitivity by targeting caveolae plasma membrane proteins, reducing insulin receptor numbers, and negatively affecting downstream insulin signaling (13, 14). Increased miR-107 expression has been described in insulin-resistant obese adult populations.

This study investigates sCD163 levels, circulating iNKT cell frequency, cytokine profile, and miR expression in obese and nonobese children.

Subjects and Methods

Study cohorts

Ethical approval was granted by the Ethics Committee, Our Lady's Children's Hospital, Dublin, Ireland. Parents of all patients gave written informed consent.

Forty-nine children (29 obese, 20 nonobese) aged 6–18 years were recruited. Subjects were categorized as obese or nonobese using International Obesity Taskforce Body Mass Index (BMI) centile charts. BMI was calculated as weight (kilograms)/height (meters)^2. Insulin (picomoles/liter), glucose (millimoles/liter), and cholesterol (millimoles/liter) levels were measured after a 12-hour fast. A clinical examination was performed in all participants, and a detailed history encompassing past medical history, current health, number and severity of previous infections, occurrence of recent infection, and use of anti-inflammatory medications was completed. Children with an underlying hormone deficiency, genetic disorder, inflammatory condition, or occurrence of recent acute infection were excluded.

Serum and peripheral blood mononuclear cell (PBMC) analysis

PBMC samples were isolated by density centrifugation as previously described (10). Cells were cultured in triplicate for 24 hours in media alone, media with lipopolysaccharide (LPS) (10 μg/mL), or media with phorbol myristate acetate (10 ng/mL) and ionomycin (5 μg/mL). Serums and cell supernatants were analyzed for cytokine, adipokine, and sCD163 levels by ELISA.

Flow cytometric analysis

PBMCs were stained for iNKT cells using BD monoclonal antibodies (6B11/CD3, BD Biosciences), as previously described (14). Cells were analyzed using FlowJo software. Cells were electronically gated (lymphogate) by their density and granularity and interrogated for the markers of interest. Results are expressed as a percentage of total T cells and determined using flow minus-1 (FMO controls).

Circulating miR analysis

Total RNA with conservation of small RNAs was extracted from PBMCs using the miRNeasy kit (QIAGEN) according to the manufacturer's instructions. Reverse transcriptase-specific primers for miR-33a, miR-33b, and miR-107 (Applied Biosystems) were used for all miR reverse transcription. Individual quantitative PCRs were carried out on the 7900HT Fast Real-time System (Applied Biosystems, Life Technologies) using miR-33a, miR-33b, and miR-107 Taqman miR probes. SnoU6 was used for normalization miR expression studies. A relative fold change in expression of the target gene transcript was determined using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$).

Statistical analyses

All analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc). Results are expressed as mean $\pm$ SD. Groups were compared using Student's *t* test or Mann-Whitney *U* test as appropriate. Parameter correlation was determined using Pearson correlation coefficients. *P* values are reported with statistical significance set at $< .05$.

Results

Weight, BMI, and metabolic parameters

Characteristics of the study participants are detailed in Table 1. Fasting glucose was similar in both groups, but there was increased insulin resistance in the obese cohort. Mean fasting cholesterol levels were elevated, and high-density lipoprotein levels were reduced in the obese cohort compared to their nonobese counterparts (Table 1).

Table 1. Characteristics of Study Subjects

	Nonobese	Obese	*P* value
n	20	29	
Gender, males/females (% male)	13/7 (65)	13/16 (45)	ns
Pubertal status, n (%)			
Prepubertal	11 (55)	9 (31)	ns
Pubertal/postpubertal	9 (45)	20 (69)	ns
Age, y	12.8 ± 3.2	13.0 ± 3.0	ns
Height, cm	147.0 ± 18.1	158.1 ± 15.4	.012
Weight, kg	43.0 ± 15.5	90.0 ± 27.5	<.001
z-BMI	0.2 ± 1.1	3.4 ± 0.5	<.001
Fasting glucose, mmol/L	4.8 ± 0.4	5.0 ± 0.4	ns
Fasting insulin, pmol/L	27.6 ± 20.20	149.0 ± 104.0	<.001
HOMA-IR	0.8 ± 0.7	4.8 ± 3.5	<.001
Cholesterol, mmol/L	3.2 ± 0.4	3.9 ± 0.7	.004
High-density lipoprotein, mmol/L	1.3 ± 0.1	1.0 ± 0.2	<.001
Low-density lipoprotein, mmol/L	1.9 ± 0.5	2.5 ± 0.6	.007
Triglycerides, mmol/L	0.7 ± 0.1	1.1 ± 0.4	<.001
Systolic BP, mm Hg	110 ± 9.5	116 ± 13.5	ns

Abbreviations: ns, not significant; HOMA-IR, homeostasis model of assessment for insulin resistance; BP, blood pressure; z-BMI, z-score for BMI. Data are expressed as mean ± SD.

sCD163 quantification

Higher levels of sCD163 were seen in the obese group (142 vs 107 ng/mL; $P = .03$) (Figure 1A), and the level correlated positively with increasing BMI ($r = 0.32$; $P = .02$) but not with fasting insulin (Figure 1, B and C).

Enumeration of iNKT cells

iNKT cell frequency was reduced within the obese cohort (0.32 vs 0.54%; $P = .001$) (Figure 1D). The change in iNKT cells was inversely correlated with sCD163 levels ($r = -0.33$; $P = .04$) (Figure 1E).

Inflammatory cytokine and adipokine profiling

Higher levels of TNF-α were detected in obese children compared to nonobese (7.2 vs 5.5 pg/mL; $P = .01$) (Figure 1F). An increase in serum leptin (1217 vs 460 pg/mL; $P < .001$) and a reduction in serum adiponectin levels (912 vs 1330 pg/mL; $P = .001$) were seen in obese children (Figure 1, G and H). PBMCs from both cohorts were stimulated with LPS for 24 hours, and increased production of IL-1β was demonstrated in the obese cohort (2108 vs 1518 pg/mL; $P = .01$) (Figure 1I).

miR profiling

There was a 4-fold increase in miR-33a expression ($P = .001$) and a 3-fold increase in miR-33b in the obese cohort compared to the nonobese ($P = .017$) (Figure 1, J and K). There was no difference in the expression of miR-107 (Figure 1L).

Discussion

Inflammation and the innate immune system play a key role in the pathogenesis of insulin resistance, T2DM, and atherosclerosis in adults (15). This study is an observational study of an exclusively Caucasian cohort of children. We show that alterations in immune cell frequency, cytokine profile, and metabolic gene regulators that characterize adult T2DM and atherosclerosis are present in obese children before the development of hyperglycemia or overt cardiovascular disease.

Obesity is associated with a chronic low-grade inflammatory state, a key driver of the adverse metabolic effects of increased adiposity. Several immune cell types, including macrophages, lymphocytes, and neutrophils, have been extensively described in adipose tissue of obese mice and humans (16). Increased numbers of circulating monocytes have been described in childhood obesity (6). M1 macrophages are abundant in inflamed adipose tissue and secrete large amounts of proinflammatory cytokines such as IL-1β and TNF-α (8). Inflammatory stimuli trigger cleavage of the scavenger receptor CD163, yielding sCD163, a marker of macrophage activation (8).

This study reports elevated levels of sCD163 in obese children, reflecting increased macrophage activation with polarization toward the M1 proinflammatory subtype. A prospective study of almost 9000 Danish adults found that increased serum concentrations of sCD163 are associated with up to five times the risk of development of T2DM, even when data are adjusted for BMI, physical activity, and lipid profile (17). Although we do report a correlation between BMI and sCD163, unlike studies in adults, sCD163 in children does not correlate with insulin resistance. This may reflect an earlier stage of the obesity inflammatory spectrum. Whether sCD163 could be used as a biomarker to prioritize children for lifestyle intervention

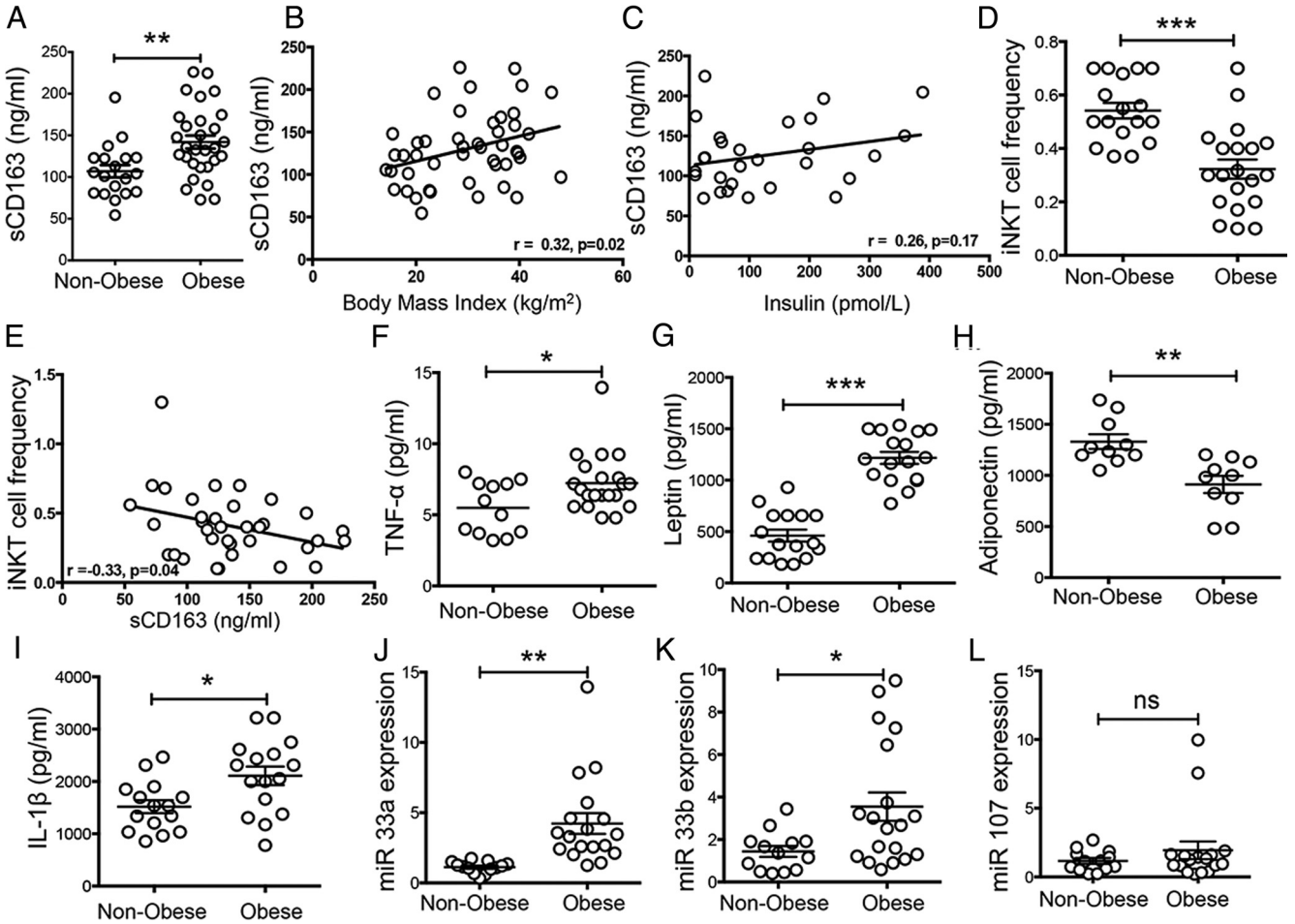

Figure 1. Inflammation, immune cell, and miR profiling in childhood obesity. A, Levels of sCD163 (ng/mL), a molecule released by proinflammatory macrophages in the serum of nonobese and obese cohorts. B and C, sCD163 levels correlate with BMI but not fasting insulin. D, Scatterplot showing the levels of circulating iNKT cells (expressed as percentage of T lymphocytes) as determined by flow cytometry; and E, their correlation with sCD163 levels in nonobese and obese cohorts. F, Scatterplot showing the levels of the proinflammatory cytokine TNF-α (pg/mL). G, Scatterplot showing levels of leptin (pg/mL) in the serum of nonobese and obese cohorts. H, Scatterplot showing the levels of adiponectin (pg/mL) in the serum of nonobese and obese cohorts. I, Scatterplot showing the levels of IL-1β (pg/mL) produced by LPS-stimulated PBMCs from nonobese and obese cohorts. Cytokines and adipokines were measured in duplicate using ELISA. J–L, Levels of miR-33a (J), miR-33b (K), and miR-107 (L) (relative expression compared to endogenous control) detected by quantitative RT-PCR in the circulating PBMCs from nonobese and obese cohorts. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

based on future risk of metabolic disease will need prospective evaluation.

This study demonstrates reduced frequency of circulating iNKT cells in obese children, with an inverse relationship between sCD163 concentration and iNKT cell number. iNKT cells are a rare subset of innate T cells that bridge innate and adaptive immunity and may act as a link between the immune and metabolic systems (10). Recent work in a murine model demonstrated that mice lacking iNKT cells had increased weight gain, insulin resistance, and M1 macrophage polarization on a high-fat diet. Adoptive transfer of iNKT cells led to decreased body fat and insulin sensitivity paired with a decrease in M1 macrophage frequency (10). The current study demonstrates the same inverse relationship between increased M1 macrophage polarization (sCD163) and decreased iNKT cell frequency in obese children. This is further evidence that the immune dysregulation seen may be contributing to the metabolic disturbances associated with obesity.

Elevated levels of proinflammatory serum cytokines have previously been described in childhood obesity (6, 7). Schipper et al (6) studied novel circulating inflammatory mediators in children aged 6 to 16 years. They reported increased levels of chemerin, IL-18, EGF, and TNF-R2 in the obese group compared to the lean group (6). We have extended this by demonstrating an altered cytokine production from PBMCs. Stimulated PBMCs from obese children produced significantly more IL-1β than those from their lean counterparts. IL-1β is released from macrophages in response to activation by large multiprotein complexes termed "inflammasomes". IL-1β plays a key role in pancreatic β-cell toxicity, progression of inflammation, and induction of insulin resistance, and thus is considered highly pathogenic in obesity-related metabolic

disease (18). Antagonism of IL-1β is currently being targeted as a possible therapeutic strategy for T2DM (19). The fact that PBMCs from obese children produce 40% more IL-1β than PBMCs from nonobese children is of concern, given their current insulin resistance and likely increased future risk of T2DM.

Upstream from cytokine protein translation, small noncoding RNAs regulate gene expression and have been implicated in fine-tuning many physiological and pathological metabolic processes. Over 1000 sequences have been described in humans, with several of these miRs identified in adipocyte biology and energy metabolism (11). MiR-33 is a key regulator in lipid homeostasis and the initiation and progression of atherosclerosis (20). This study demonstrates an increase in both miR-33a and miR-33b in obese children—miRs that are known to inhibit generation of high-density lipoprotein cholesterol and reduce cellular fatty acid oxidation (20). MiR-107, implicated in insulin resistance in adults, is similar in the obese and nonobese cohorts. This suggests that some of the processes underpinning insulin resistance may not yet be under way at this age and highlights the importance of early intervention even in established childhood obesity. The identification of dysregulated miRs may ultimately provide biomarkers that support targeted interventions to those most at risk of complications.

A limitation of this study is that the cohorts were not matched for pubertal status or insulin resistance. Further studies will be required to determine the impact of both on the clear differences we have observed between the obese and nonobese cohorts.

In summary, childhood obesity associated with insulin resistance impacts on the immune system at multiple levels, including cytokine production, macrophage polarization, innate immune cell frequency, and regulation of gene expression. These changes have been causally linked to the onset of metabolic disease in adulthood and suggest the future trajectory of obese children to the development of T2DM and premature cardiovascular disease.

Acknowledgments

We are indebted to the children and families who took part in our study and to the endocrine nursing staff at Our Lady's Children's Hospital, Crumlin for all their help and support.

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E.C. and A.E.H. are supported by the National Children's Research Centre. The Obesity Immunology Group is supported by the Irish Health Research Board, National Children's Research Centre, Ireland, and UCD Newman Fellowship Programme. N.F. and L.A.O. are supported by a European Research Grant. E.C. and A.E.H. are supported by the National Children's Research Centre, Ireland.

Disclosure Summary: The authors declare no conflict of interest.

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