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Canadian Study of Determinants of Endometabolic Health in ChIlDrEn (*CanDECIDE study*): a cohort study protocol examining the mechanisms of obesity in survivors of childhood brain tumours

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ABSTRACT

Background: Childhood obesity has reached epidemic proportions and is impacting children's health globally. In adults, obesity is associated with chronic low-grade inflammation that leads to insulin resistance, which is one of the important mechanisms through which dysregulation of metabolism occurs. There is limited information available about the contribution of inflammation to metabolic health in obese children, and how individual and lifestyle factors impact this risk. One of the paediatric groups at risk of higher rates of obesity includes the survivors of childhood brain tumours. The aim of this study was to evaluate the mechanisms that contribute to inflammation in obese survivors of childhood brain tumours.

Methods and analysis: This is a prospective cohort study. We will recruit lean and obese survivors of childhood brain tumours, and a control group composed of lean and obese children with no history of tumours. We will measure circulating and urinary cytokine levels and cytokine gene expression in monocytes. In addition, the methylation patterns of cytokine genes and that of toll-like receptor genes will be evaluated. These will be correlated with individual and lifestyle factors including age, sex, ethnicity, puberty, body mass index, fasting lipid levels, insulin sensitivity, diet, exercise, sleep, stress and built environment. The sample size calculation showed that we need 25 participants per arm

Ethics and dissemination: This study has received ethics approval from the institutional review board. Once completed, we will publish this work in peer-reviewed journals and share the findings in presentations and posters in meetings.

Discussion: This study will permit the interrogation of inflammation as a contributor to obesity and its complications in obese survivors of childhood brain tumours and compare them with lean survivors and lean and obese controls with no history of tumours, which may help identify therapeutic and preventative interventions to combat the rising tide of obesity.

ARTICLE SUMMARY

Article focus

- The goal of the Canadian Study of Determinants of Endometabolic Health in Children (CanDECIDE) study is to determine if obese survivors of childhood brain tumours have more inflammation than lean survivors, and lean and obese children with no history of tumours.
- It will also evaluate the potential mechanisms involved in the occurrence of inflammation and immune system activation in these groups.

Key messages

- This study will determine the inflammation and immune system activation status in obese and lean survivors of childhood brain tumours and compare them to lean and obese children with no history of tumours.
- This may allow the determination of preventative and therapeutic strategies to mitigate the risk of obesity and its comorbidities in this population.

Strengths and limitations of this study

- The strength of this study is that it will systematically study the inflammatory response in childhood obesity in paediatric brain tumour survivors.
- A potential limitation is that measuring the inflammatory response in the basal state may not demonstrate differences, and ligands to stimulate the inflammatory response in cells in vitro will be used if this is the case.

BACKGROUND

Childhood obesity: an epidemic of global proportions

Obesity affects around 1.5 billion people around the globe today,[1–3] and of those 200 million are children.[2 4–6] In Canada, the rates of childhood obesity have tripled over

the past two decades, and currently around 25% of children are overweight or obese,[7] with certain ethnic groups including aboriginal and South Asian communities bearing the brunt of the epidemic with rates of around 40%.[8 9]

Obese children have a higher chance of developing obesity-related complications including hypertension, non-alcoholic fatty liver disease, dyslipidaemia and type 2 diabetes during childhood. In addition, obese children are likely to become obese adults,[10–13] increasing their risk of type 2 diabetes and cardiovascular disease.[2 14] These children are developing diseases of adults at an ever younger age, defining obesity as a state of premature ageing that impacts the longevity and quality of life of a generation that will live with obesity and its comorbidities for decades, as they are likely to live longer.

In addition to the above cardiometabolic complications, adult obesity is associated with increased risk of certain cancers and may impact treatment outcomes.[15] An important question is whether childhood obesity is associated with increased risk of cancer in children and adults, as some obese adults were obese as children. There is recent evidence that childhood obesity is associated with increased risk of adult colon and urothelial tumours.[16 17] What is not clear so far is if childhood obesity increases the risk of tumours during childhood, as well as its potential effect on the long-term metabolic outcomes in those who survive childhood cancer.

In addition, some childhood tumours and their treatment increase the risk of obesity and its comorbidities in survivors, and one such group of patients is survivors of childhood brain tumours.

Survivors of childhood brain tumours have a higher risk of obesity

Brain tumours are the second commonest cause of death in children after accidents.[18] Over the past four decades, novel diagnostic neuroimaging modalities coupled with therapeutic advances have led to a significant reduction in mortality.[19 20] As survival rates improved, it became apparent that these patients have higher premature mortality[21] and morbidity rates, and one such morbidity is obesity.[22–28] The aetiology of obesity in brain tumour survivors is polygenic and can be due to the tumour and its treatment interacting with the patient's genetic, epigenetic and environmental factors. In some tumours, including gliomas, the presence of obesity is a marker of poor prognosis.[29]

Brain tumours can cause damage to the hypothalamic-pituitary region due to their location and size, with pressure and infiltration of the surrounding structures. In addition, injury to the hypothalamic-pituitary structures can be secondary to chemotherapy,[30] or due to radiotherapy when the structures are in the path of radiation beams, or when tumours are adherent to the surrounding structures and being removed surgically. Damage to the ventromedial hypothalamus impairs satiety/hunger signalling by leptin, ghrelin and insulin, all of which have hypothalamic receptors leading to hyperphagia. In addition, hypothalamic damage slows the basal metabolic rate and causes an increased parasympathetic tone, which increases insulin secretion and enhances lipogenesis contributing to weight gain.[31–33]

In addition, obesity in survivors may be related to deficiency of hypothalamic hormones including growth hormone-releasing hormone, thyroid-releasing hormone or gonadotropin-releasing hormone[31 32 34] or damage to the pituitary stalk, which prevents these peptides from reaching the pituitary gland. Alternatively, the production of the pituitary hormones may be impaired due to direct pituitary gland damage that may lead to impaired production of growth hormone, thyroid stimulating hormone and gonadotropins.[32 34 35]

Other factors that contribute to obesity include limited mobility and reduced physical activity.[36] This may be related to reduced exercise capacity due to complications of therapy including pulmonary fibrosis secondary to thoracic irradiation,[37] or cardiac disease due to the effects of chemotherapy or radiation on the heart,[38] sleep problems related to hypothalamic damage,[39] vision problems as well as neurosensory and mobility problems and pain.[22 40] It may also be related to psychological or cognitive dysfunction, or may be facilitated by the way the child is perceived to have different exercise tolerance and ability to handle physical activity and is allowed to develop sedentary habits, for example, watching TV.[23 28 41] Furthermore, some of the drugs used in these patients during and after their brain tumour treatment are obesogenic including steroids, antidepressants, antipsychotics and antiepileptic medications.[42]

In addition to obesity increasing the risks of metabolic disease, survivors of childhood cancer have an increased 30-year risk profile for myocardial infarction, stroke and coronary death whether or not they received cardiotoxic therapy.[43] Importantly, non-high density lipoprotein, insulin levels and high C reactive protein (CRP) were elevated when compared with non-cancer controls. This indicates that cancer itself is associated with the pathogenesis of adverse cardiometabolic outcomes in these patients and obesity may add to this risk.[43]

New insights into causation of obesity: immune system activation and inflammation

Over the past few years, further understanding emerged regarding the mechanisms of obesity, and one such mechanism is inflammation. Obesity is coupled with chronic low-grade inflammation that starts in the adipose tissue.[44] Hypertrophy and hyperplasia of the adipose tissue is characterised by local tissue hypoxia and activation of the inflammatory response, with secretion of inflammatory molecules called cytokines leading to local inflammation.[45]

A major source of inflammatory cytokines in obese adipose tissue is an immune cell, the macrophage,[44 46] but other immune cells including neutrophils and T

lymphocytes that are either present in or arrive at expanding adipose tissue also contribute to this process.[47–53]

In addition to cytokines, saturated fatty acids provide another pathway for induction of obesity-mediated inflammation. Saturated fatty acids are taken by the adipose tissue during the development of obesity and are stored in adipocytes. When fatty acid supply exceeds the adipose tissue storage capacity, they spill into the circulation and reach remote metabolic organs including the skeletal muscle and liver.[54–56]

Fatty acids exert their effects in two different ways. They can bind to receptors present on the surface of immune and metabolic cells called Toll-like receptors (TLRs) that include TLR2 and TLR4 and initiate signalling through the receptor and its signalling pathway.[57 58] Alternatively, fatty acids may be transported intracellularly and metabolised to generate lipid intermediates including ceramides and diacylglycerol.[54 59]

Both inflammatory cytokines and fatty acids and their metabolites collaborate to trigger the activation of intracellular inflammatory pathways including mitogen-activated protein kinases, protein kinase C and inhibitor of nuclear factor-κB kinase-β.[49]

The activation of these pathways will stimulate further cytokine production, leading to inhibition of insulin signalling and insulin resistance in metabolic organs.[60] With insulin resistance, a compensatory increase in endogenous insulin production ensues, leading to hyperinsulinaemia. When pancreatic insulin production fails to keep up with demand, type 2 diabetes develops.[61 62]

Innate immunity, macrophage phenotypes and immunometabolic interactions in obesity

The innate immune system is the initial line of protection against environmental threats. Its cells are present at ports of entry of pathogens and toxins to the body, and their activation occurs very shortly after exposure. If innate immune responses are not sufficient to combat the threat, then adaptive immunity is activated.[62]

Over the past few years, evidence has been accumulating for a role of the innate immune system in obesity, with its cells, pathways and molecules intertwined with those in metabolic organs.[49 63 64]

Some of the innate immune system components include immune cells like monocytes and neutrophils and receptors including TLRs noted earlier. In addition to the production of cytokines, the inflammatory response in obesity is associated with the production of molecules called chemokines that help direct leucocytes into metabolic organs. Through the actions of chemokines, circulating monocytes are attracted to metabolic organs and differentiate to macrophages, which are present in two main subtypes.

Inflammatory or 'M1' macrophages originate from bone marrow-derived monocytes that enter the expanding adipose tissue. These cells produce inflammatory cytokines and are detected in fat, skeletal muscle and the liver.[47 65 66]

The anti-inflammatory or 'M2' macrophages are resident macrophages that exist under physiological conditions and help with tissue homeostasis and remodelling, as well as reducing adipose tissue inflammation in obesity.[67]

Monocytes recruited during weight loss are another source of M2 macrophages, which help with the processing of fatty acids in adipose tissue during this phase; the numbers of these cells drop once weight loss is achieved.[68] The loss of anti-inflammatory actions of M2 macrophages and augmented inflammatory responses by M1 macrophages is considered to be a central driver of the adverse effects of inflammation in obesity.[69]

Animal and adult human studies clearly document the presence of inflammation and the activation of innate immunity in obesity.[70–72] On the other hand, little systematic inquiry has been carried out to elucidate the immunometabolic interactions in childhood obesity,[73–75] and how this shapes the landscape in children for future metabolic risk. One study reported that obese children had a higher number of circulating monocytes when compared with lean children.[76] Few papers have shown evidence of elevation of inflammatory markers in obesity.[77–80]

It is important to understand the association between inflammation, obesity and cancer as some of the mechanisms that may be 'hard-wired' in adults may still be amenable to modification in children. We know nothing about these mechanisms and their potential long-term reversibility in children, and this is also the case for survivors of childhood brain tumours.

Local versus systemic inflammation in cancer

Local chronic inflammation in cancer is a well-established paradigm. Cancer is initiated by exposure to stimuli, which leads to DNA and other cellular changes. These changes do not harm the host until a promoter is encountered,[81] which can include chemicals, pathogens, hormones, growth factors or cytokines.

The end result is dysregulation of cell death and repair mechanisms coupled with production of reactive oxygen species and unregulated cellular growth. Tumour cells secrete a mix of cytokines and chemokines that contribute to local inflammation and immune cell attraction.

The immune cells that are present in tumours include monocytes, neutrophils, eosinophils, dendritic cells, lymphocytes and mast cells.[81] These cells are bathed in cytokines, nutrients and growth factors that constitute the tumour microenvironment, and they in turn start secreting their own array of cytokines in response to their environment. Both tumour and immune cells contribute to the creation of the tumour microenvironment that regulates tumour cell growth, invasion, metastasis and metabolism. The latter also regulates the differentiation of macrophages that associate with tumours called tumour-associated macrophages (TAM).[82]

TAM play a critical role in defining the tumour microenvironment and tumour progression as they play a dual role by not only killing neoplastic cells but also secreting angiogenic and lymphangiogenic factors and cytokines that help the proliferation of tumours. In addition, they secrete interleukin (IL)-10, which ameliorates the function of cytotoxic T lymphocytes responsible for tumour killing. These cells have been detected in brain tumours and their role in local inflammation is evolving,[83 84] and it is unclear if they play a role in systemic inflammation in these patients.

The presence of systemic inflammation in paediatric brain tumours is not well studied. In a recent report, survivors of childhood tumours, including a small group of brain tumour survivors, were demonstrated to have hyperinsulinism, dyslipidaemia and elevated CRP.[43] This was independent of treatment status, which argues for a direct role of the tumour itself or perhaps its immune cell complement in affecting systemic metabolism and inflammation. Whether this is the case, or indeed if these cells leave an inflammation signature even after tumour treatment that alters local or systemic inflammation and metabolism, is unknown.

It is also unclear if the additional obesity risk factors in survivors will increase the risk of having more inflammation when compared with lean survivors, and there are no studies that have interrogated the connection between immunity and metabolism in survivors of childhood brain tumours.

Understanding the mechanisms of immunometabolic interactions in obesity in this group may allow the design of effective treatment and prevention programmes to combat obesity and its complications, so that survival is not accompanied by an increased burden of comorbidities.

DNA methylation and regulation of gene transcription

The expression of cytokine genes requires them to be accessible to the transcription machinery of the cell. Transcription factors including the polymerase enzyme form the transcription machinery that bind to the gene promoter region, and start copying the gene to produce a complimentary copy of the DNA called messenger RNA. The latter is then used to synthesise the cytokines in the ribosomes.

Methylation is one mechanism by which DNA transcription is regulated. It involves adding a methyl group to 5-carbon on cytosine residues in the CpG dinucleotide area in the gene promoter. The methylation status of a gene is important in determining its transcription, and new understanding indicates that the spatial location of methylation is important in activating or silencing gene transcription.[85] We have no knowledge of the role of cytokine gene methylation in inflammation in obese survivors of childhood brain tumours (OBT) or their obese controls with no history of tumours.

As obesity and cancer are inflammatory states, and as survivors of childhood brain tumours have multiple factors that contribute to a higher risk of obesity, the aim of this study is to determine if OBT have enhanced inflammation when compared to lean survivors of childhood brain tumours (LBT), obese, children with no history of tumours (ONB) and lean children with no history of tumours (LNB).

HYPOTHESES

Primary hypothesis

OBT have more enhanced inflammation when compared with LBT, LNB, and ONB groups.

Secondary hypothesis

The enhanced inflammatory response seen in OBT when compared with LBT, LNB, and ONB groups is mediated via individual and lifestyle factors.

OBJECTIVES

Primary objectives

1. To determine inflammatory cytokine levels in OBT, LBT, LNB, and ONB groups.
2. To quantify monocyte TLR and inflammatory cytokine gene expression in OBT, LBT, LNB, and ONB groups.
3. Determine methylation patterns of the monocyte TLR pathway and inflammatory cytokine genes in OBT, LBT, LNB, and ONB groups (table 1).

Secondary objectives

To determine the relation between diet, physical activity, adiposity, sleep, stress and built environment and cytokine levels, gene expression and DNA methylation patterns of TLR and cytokine genes in OBT, LBT, LNB, and ONB groups (table 2).

Study methods and design

This is a prospective cohort study. We will recruit participants from clinical services within our institution. Follow-up of participants will continue for 10 years at 2 yearly intervals. The study flow chart is shown in figure 1. Ethics approval has already been obtained from the institutional Research Ethics Board.

Eligible participants include children who are 5 years and older, and who are lean (body mass index (BMI) below the 85th centile for age and gender), overweight (BMI between the 85th and 95th centiles for age and gender) and obese (BMI above the 95th centile for age and gender). In addition, potential participants should have no known history of viral, bacterial or fungal infections over the 2 weeks prior to participation, and no history of autoimmune diseases. In relation to medications, participants should have not received immunosuppressive therapy or systemic steroids that are higher than maintenance levels (6–8 mg/m^2/day), or inhaled steroids that are above the standard doses recommended for asthma therapy for 15 days prior to participation. In addition, for those with a history of brain tumours, they

Table 1 CanDECIDE study primary objectives and statistical analysis plans

Objectives	Outcome	Independent variables	Hypotheses	Statistical analysis
1. Measurement of cytokine levels 2. Gene expression of TLR and cytokine genes 3. DNA methylation patterns of TLR and cytokine genes In obese survivors of childhood brain tumours and comparing them with non-obese tumour survivors and lean and obese patients with no history of tumours	1. Cytokine levels 2. TLR and cytokine gene expression (RNA) 3. DNA methylation patterns of TLR and cytokine genes Determine the presence of inflammation and the mechanisms involved in its development in obese survivors of childhood cancer vs controls	1. Age 2. Sex 3. Ethnicity 4. Puberty 5. Tumour type 6. Tumour location 7. Tumour treatment 8. Hormonal deficiencies 9. BMI 10. Lipid levels 11. HOMA-IR	Obese survivors of childhood brain tumours have higher inflammatory status and altered gene methylation patterns compared with lean survivors and non-cancer controls that predispose them to endometabolic risks	Regression

BMI, body mass index; CanDECIDE,Canadian Study of Determinants of Endometabolic Health in ChIlDrEn; TLR, Toll-like receptor.

should have completed therapy for at least 6 months prior to enrolment. Exclusion criteria include a history of smoking, active infection, autoimmune disease or inability or refusal to provide consent. These inclusion and exclusion criteria will apply throughout the study duration.

INFORMED CONSENT

On the day of clinic, the clinic staff will ask potential participants and parents for permission to be approached so that further information can be provided about the study. If the patient or parent gives permission, the researcher will collect contact details and data including age and gender. They will also explain the study and provide information brochures along with the team's contact details.

If the family and participant agree to join the study, the researcher will schedule an appointment for a dedicated research clinic visit within 4 weeks. These include parent or participant consent forms if the latter is 16 years or older, assent forms for those between 7 and 15 years of age, and separate consent form for genetic (DNA) testing.

DEDICATED RESEARCH CLINIC VISIT

On the day of the visit, the researchers will check that participants are fasting and will direct them to the consent explanation and signature station. This is

Table 2 CanDECIDE study secondary objectives and statistical analysis plans

Objectives	Outcome	Independent variables	Hypotheses	Statistical analysis
Secondary determination of role of 1. Diet 2. Physical activity 3. Adiposity 4. Sleep 5. Stress 6. Built environment That impacts circulating cytokine levels, gene expression and DNA methylation patterns of TLR and cytokine genes in obese survivors of childhood brain tumours and comparing them with non-obese tumour survivors and lean and obese patients with no history of tumours	Understand the role of 1. Diet 2. Physical activity 3. Adiposity 4. Sleep 5. Stress 6. Built environment On development of inflammation in obese survivors of childhood cancer and comparing them with non-obese tumour survivors and lean and obese patients with no history of tumours	1. Age 2. Sex 3. Ethnicity 4. Puberty 5. Physical activity 6. Tumour type 7. Tumour location 8. Tumour treatment 9. Hormonal deficiencies 10. BMI 11. Lipid levels 12. HOMA-IR	Obese survivors of childhood brain tumours have higher inflammatory status and altered gene methylation patterns, and this is mediated via individual and lifestyle factors	Descriptive analysis Regression

BMI, body mass index; CanDECIDE,Canadian Study of Determinants of Endometabolic Health in ChIlDrEn; TLR, Toll-like receptor.

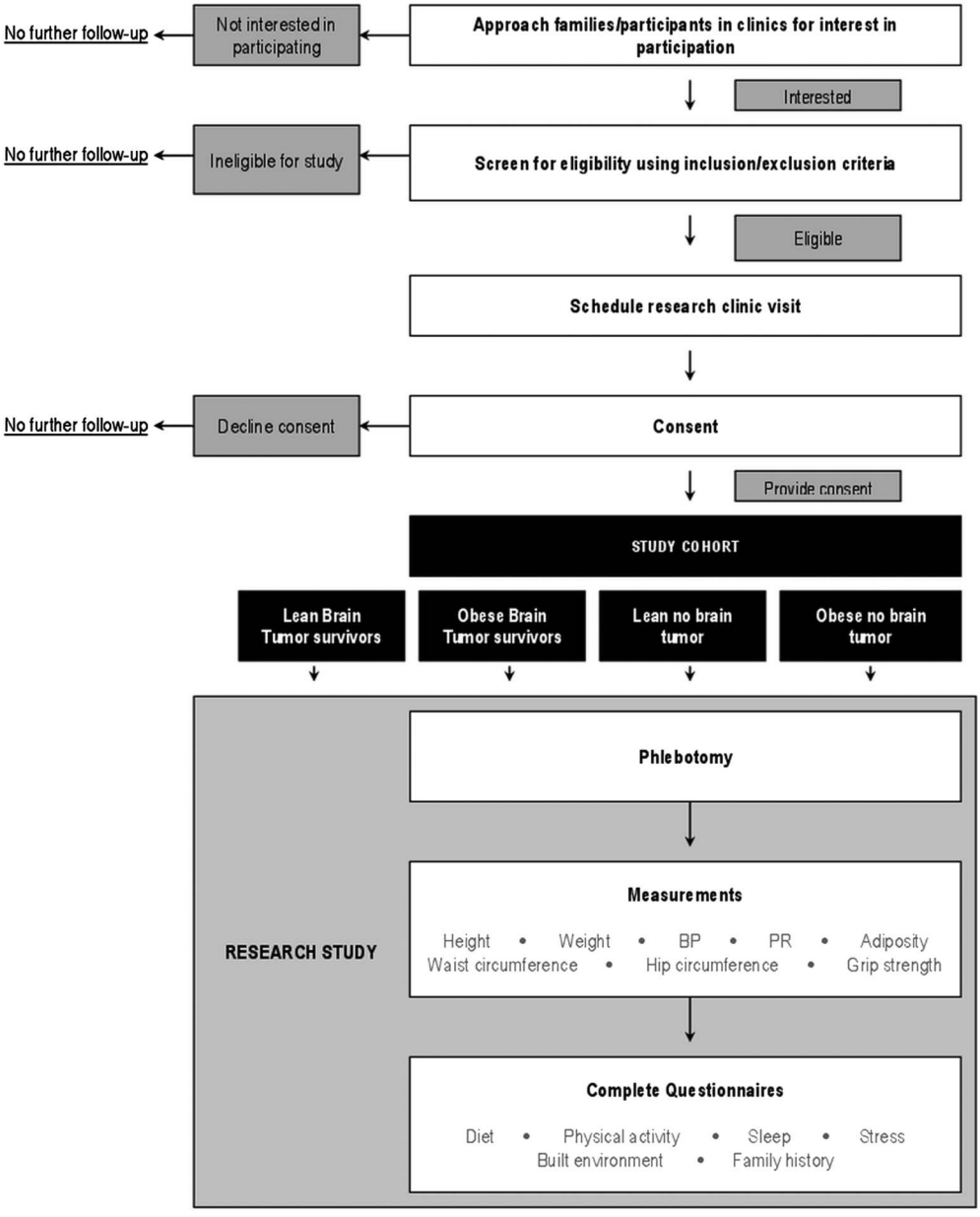

Figure 1 Flow chart of the Canadian Study of Determinants of Endometabolic Health in ChIlDrEn (CanDECIDE) study. The potential participants will be approached during their routine clinic visits to determine if they are interested in participating. If so, a dedicated research clinic visit will be conducted for consenting and enrolment. The participants will be stratified into the four arms of the study including obese childhood survivors of brain tumours (OBT), lean childhood survivors of brain tumours (LBT), obese children with no history of tumours (ONB) and lean children with no history of tumours (LNB). Anthropometric, adiposity and grip strength measurements and completion of questionnaires will be completed during that visit. In addition, blood, saliva and urine samples will be collected.

followed by a visit to the phlebotomy station for blood sample collection and the provision of containers to obtain saliva and urine samples. Samples will be processed within 120 min of collection, and the samples will be promptly anonymised using unique identifying numbers to protect confidentiality.

MEASUREMENTS

The participant height will be measured closest to 0.1 cm using a stadiometer, weight closest to 0.1 kg using a weighing scale, and BMI in kg/m^2 calculated from height and weight. Central adiposity will be determined by measuring waist circumference and hip circumference using a spring-loaded measuring tape closest to 0.1 cm. Sitting right arm systolic and diastolic blood pressure (BP) and pulse rate will be measured twice using an automated BP and heart rate monitor. Total adiposity will be measured by quantifying body fat percentage using the Tanita body fat monitor for children (Tanita Corporation, Illinois, USA), and muscle strength will be tested using a dynamometer.

QUESTIONNAIRES

We will collect sociodemographic data including age, gender, school grade or job description, parental education, parental reported height and weight, religion, ethnicity, birth history, family history, feeding and vaccinations from all participants. In addition, tumour type, tumour location, treatment protocol, complications of tumour or its therapy, history of medical or surgical problems and current medications including vitamin supplements will be collected for survivors of childhood brain tumours. For participants below 12 years of age, the parent will fill this questionnaire, while the participant and parent will fill it if they are 12 years or older. To assess pubertal stage,[86] we will use line drawings depicting Tanner pubertal staging for breasts in girls 8 years or older, and for the external genitalia for boys 9 years and older.

Diet

Regarding dietary intake, we will use items from the Youth and Adolescent Food Frequency Questionnaire, which had recently been updated.[87 88] This is a questionnaire developed in a US paediatric cohort, and includes questions about food intake based on average portion sizes of different dietary constituents. In addition, questions about sugary drinks and eating behaviours are also collected. The number of servings per day will be calculated from the questionnaire by multiplying the frequency of consumption by portion size.[89]

Physical activity

We will measure physical activity using the Habitual Activity Estimation Scale (HAES) questionnaire,[90] which is used to measure physical activity in the paediatric population. The participant is asked to report the percentage of time spent at different levels of activity (inactive, somewhat inactive, somewhat active and very active) on one weekday and one Saturday, and examples of the types of activities are given with the questionnaire. The time percentages will then be converted to minutes per day and used in the analyses.

The data will be pooled so that two groups are created, including those with 'inactive' and 'somewhat inactive' designation and those with 'somewhat active' and 'very active' groups.

HAES has been used in healthy paediatric populations and those with chronic disease and correlates well with accelerometer data, as well as having high test–retest reliability.[91] Furthermore, we will also document motor disabilities while conducting these assessments.

Sleep

We will measure sleep duration and quality using a standardised paediatric sleep questionnaire,[92] which correlates highly with polysomnography and has a sensitivity of around 85% and specificity of 87% to detect sleep-disordered breathing, and high test–retest reliability.[92]

Stress

We will also enquire about mental health issues using a questionnaire reporting mood disorders.[93] We will screen for depressive symptoms using the Center for Epidemiological Studies Depression Scale for Children, which is another validated tool to screen for mental health issues including depression. This item is scored from 0 to 60, and scores above 15 warrant further evaluation.[93]

Built environment

We will evaluate the built environment using the Neighborhood Environment Walkability Scale, which is a validated tool that measures factors related to neighbourhood design, access and safety.[94] These scores can then be correlated to physical activity levels and metabolic parameters.

BLOOD SAMPLING

Blood samples will be collected at the start, at 4 years and the end of the study. Certified phlebotomists in the hospital or trained healthcare professionals will take fasting blood samples. These samples include serum, plasma, complete blood counts and samples to isolate white blood cells (leucocytes). The total volume of blood needed is around 20 mL. Saliva and urine samples will also be taken for DNA analysis and measurement of cytokines, respectively.

We will process and isolate the appropriate analytes, and freeze samples at −80°C until further analysis.

URINE SAMPLING

Urine samples will be collected at the start, at 4 years and the end of the study. Participants will provide a urine sample in 90 mL plastic containers, and aliquots will be frozen at −80°C until further analysis.

SALIVA SAMPLING

Participants will provide a saliva sample in the Oragene DNA saliva collection kit. Samples are stored at room temperature until further analysis.

EXPERIMENTAL WORK

Determination of circulating and urinary cytokine levels

The study will have four arms including OBT, LBT, LNB, and ONB groups.

We will quantify cytokine concentrations in the serum and urine using the Multiplex ELISA kits (tumour necrosis factor α (TNFα), IL-1β, IL-6, CCL-2, IL-8, IL-10, IL-18, MIP-1α). We chose these cytokines as they represent known markers of inflammation (TNFα, IL-1β, IL-6, IL-10, IL-18) and immune cell attraction (CCL-2, IL-8, MIP-1α) to different tissues.

Quantification of monocyte TLR and cytokine gene expression

We will isolate monocytes from peripheral blood using monocyte enrichment kits (Stem Cell Technologies) as per the manufacturer's instructions. Cells will be sorted based on the monocyte markers CD14 and CD16.

We will isolate RNA using the RNAeasy minikit (Qiagen). Quantification and determination of purity will be carried out using a nanospectrophotometer. The SuperScript III reverse transcriptase kit (Invitrogen) will be used to generate cDNA as per the manufacturer's instructions. We will use TaqMan probes (Applied Biosystems) to measure the gene expression status of cytokines (TNFα, IL-1β, IL-6, CCL-2, IL-8, IL-10, IL-18, MIP-1α) by Quantitative Real-Time PCR (qRT-PCR).

The TLR pathway gene expression profiling looking at 84 genes involved in the TLR expression and signalling pathway will be carried out using the Human Toll-Like Receptor Signalling Pathway PCR Array (SABiosciences) as per the manufacturer's instructions.

Determination of methylation patterns of the monocyte TLR pathway and cytokine genes

We will isolate genomic DNA from monocytes using the DNAeasy Mini kit (Qiagen) as per the manufacturer's instructions. The DNA will then be processed for qRT-PCR reaction using the SYBR Green reaction master mix to measure the methylation patterns for the cytokine (TNFα, IL-1β, IL-6, CCL-2, IL-8, IL-10, IL-18, MIP-1α) and TLR genes.

Sample size calculation

The clinical services from which participants will be recruited include the Neurooncology, Orthopaedics and Cardiology services. The Neurooncology programme at our institution cares for 270 survivors of childhood brain tumours and the programme reviews patients annually or more frequently depending of the tumour type and time from completion of therapy, with clinics serving 8 patients/week. The Orthopaedics clinic serves 70–80 patients/week and the Cardiology service performs assessments of 24 patients/week. Assuming a 50% recruitment rate based on fulfilment of inclusion criteria and interest in participation, our goal is to recruit 150 OBT & LBT and 150 LNB & ONB: at a rate of 3 patients/week over a 2-year period.

On the basis of these figures, we estimate to have 99.7% power to reject the null hypothesis with α set at 0.05, variable in the pooled analysis to clarify its association with inflammation in survivors. This is based on an earlier study that found a difference in cytokine CCL2 levels between lean and obese children.[80] Importantly, to obtain 80% power, we will need 25 participants in each of the groups, and these calculations are performed using the Power and Sample Size Calculations software V.3.0.43.[95] Our overall recruitment target is consistent with the reported enrolment rates of children with cancer in clinical trials, with rates ranging from a 70% participation rate in the 0-year-old to 14-year-old group[96] and dropping to 24% in the 15-year-old to 19-year-old category, and other earlier studies demonstrating even lower rates of recruitment in the latter age group.[97–99]

There is conflicting evidence regarding the participation of ethnic minorities in paediatric cancer studies, with some showing appropriate representation and others documenting underrepresentation.[100–102] We are including all ethnic groups and will monitor this aspect of recruitment closely as our intention is to investigate a representative sample of children.

Statistical analyses

The analysis results of patients' demographics and baseline outcome variables (both primary and secondary) will be summarised using descriptive summary measures expressed as the mean (SD) or median (minimum–maximum) for continuous variables and number (per cent) for categorical variables. In addition, we will test for differences in sociodemographic and baseline clinical characteristics between groups using χ^2 tests for categorical variables and one-way analysis of variance or Kruskal-Wallis tests for continuous variables depending on the distribution.

All analyses of primary and secondary outcomes will be performed using regression analysis to compare the groups adjusting for age, sex and ethnicity. The results will be reported as estimates of the difference, corresponding to 95% CI and associated p values. Statistical significance will be set at α=0.05 adjusted using the Bonferroni approach for multiple analyses. We will examine the residuals to assess model assumptions. All analyses will be performed using the SAS V.9.2 (Cary, North Carolina, USA) or SPSS (Chicago, Illinois, USA) statistical software.

The majority of tumours in our patients are divided into three groups including gliomas, medulloblastomas and ependymomas. Other less common tumours include PNET, and a mix of germ cell tumours, germinoma, atypical neurocytoma, ATRT and other rare tumours. We will therefore plan subgroup analyses based on three groups to ensure adequate power to detect statistical differences. In addition to the subgroup analysis, we will also include the tumour type as an independent variable in the pooled analysis to clarify its association with inflammation in survivors.

Other factors that may have an impact on obesity such as cancer therapy, hormonal abnormalities and impaired mobility will be taken into account as confounding variables in regression analyses. Regression analysis allows the inclusion of several variables as explanatory factors, and therefore we will not perform subgroup analyses using these variables.

DISCUSSION

In this study, we will investigate the mechanisms of inflammation in OBT and compare them to LBT, LNB, and ONB groups.

The documentation of monocyte activation status in OBT children is critical, as these cells play a fundamental role in generating and propagating inflammation in different tissues, and are involved in atherosclerosis and diabetes development. If these cells are already activated at the paediatric age group and express inflammatory markers, then interventions may have to be more aggressive including lifestyle intervention, nutraceutical and pharmacological treatments to address these mechanisms.

Understanding the methylation status of TLR in OBT children is essential, as discovering the methylation patterns of different genes will clarify which genes are activated or silenced in the TLR pathway. If there are differences between groups, the next step is to conduct a randomised controlled trial using nutraceutical of pharmacological therapy with or without lifestyle intervention to identify the most effective intervention(s) in ameliorating inflammation, and if this occurs via modulation of the methylation patterns of cytokine and TLR genes.

As this study is at the inception phase, we are not certain that measured inflammation in this group is different from that in the lean survivors or lean and obese children in the non-tumour group. We are testing gene expression of inflammation markers in the basal state, and it may be necessary to stimulate those cells with cytokines, lipopolysaccharide or fatty acids to illustrate responses to the obesogenic environment they are exposed to in vivo. It may be that when cells are challenged in vitro, they may elicit responses that otherwise will not be apparent. We may also use TLR ligands to stimulate cells and measure the TLR gene expression pathways. This may help elucidate responses to the obesogenic environment these cells inhabit.

This work will enrich our understanding of the mechanisms of inflammation in childhood obesity in survivors of childhood brain tumours, as well as the lifestyle and environmental factors that impact these mechanisms. This may allow the development of targeted therapeutic and preventative strategies to deal with inflammation in obesity and its comorbid associations.

As this study is using lean and obese children with no history of tumours as controls, this will allow us to study inflammatory cytokine and TLR gene expression and cytokine levels in children, and define the different life style factors that may mediate the interaction between adiposity and activation of these pathways in immune cells in children. This will add significantly to our understanding of the interaction between immunity and metabolism in the general pediatric population.

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