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Determinants of Endometabolic Health in ChIldrEn (*DECIDE study)*: A cohort study protocol examining the mechanisms of obesity in survivors of childhood brain tumors

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Abstract:

Introduction: 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 pediatric groups at risk of higher rates of obesity includes the survivors of childhood brain tumors.

The aim of this cohort study is to evaluate the mechanisms that contribute to inflammation in obese survivors of childhood brain tumors.

Methods & analysis: This is a prospective cohort study. We will recruit lean and obese survivors of childhood brain tumors, and a control group composed of lean and obese children with no history of tumors. The groups will be evaluated for their inflammatory profile including the measurement of circulating and urinary cytokine levels and cytokine gene expression in monocytes. In addition, methylation patterns of cytokine genes and that of toll-like receptor genes will be evaluated. These will be correlated with individual factors including age, sex, ethnicity, puberty, body mass index, fasting lipid levels, and insulin sensitivity.

Sample size calculation showed that we need 29 participants per arm.

Ethics & 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 tumors and comparing them to lean survivors and lean and obese controls with no history of tumors, which may help identify therapeutic and preventative interventions to combat the rising tide of obesity.

Keywords: Obesity, brain tumor, Immunometabolism, inflammation, Toll-Like Receptors, cytokines

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 [4]. In Canada, the rates of childhood obesity have tripled over the past two decades, and 25% of children today are overweight or obese [5 6], with certain ethnic groups including aboriginal and South Asian communities bearing the brunt of the epidemic with rates of around 40% [7 8]. Obese children have a higher chance of developing obesity-related complications including chronic diseases like type 2 diabetes. In addition, obese children are likely to become obese adults [9-12], and this increases their risk of diabetes and cardiovascular disease [2 13]. These children are developing diseases of adults at an ever younger age, making obesity a state of premature aging that impacts the quality of life and life span of a generation of children who will live with obesity and its comorbidities for decades, as they are likely to live longer.

Evidence links obesity with increased risk of certain tumors and may also impact tumor treatment outcomes in adults [14]. Recent indications are linking childhood obesity with increased risk of adult colon and urothelial tumors [15 16]. What is not clear so far is the influence of childhood obesity on the probability of tumors during childhood and its potential effect on long-term metabolic outcomes.

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

Survivors of childhood brain tumors have a higher risk of obesity

Brain tumors are the second commonest cause of death in children after accidents [17]. Recent therapeutic advances have resulted in a significant reduction in mortality, but survivors face a number of metabolic morbidities, including obesity, that impact their overall health [18-21] and this may be related to the tumor or its management.

The two commonest brain tumors are low-grade gliomas and medulloblastomas. The former carries significant morbidity rates following its treatment including neuroendocrine sequelae and hormonal defects that can become apparent years after the completion of treatment [22 23]. The presence of obesity in these patients is a marker of poor prognosis [24].

Understanding the fundamental mechanisms of obesity development in this group will allow the design of effective treatment and prevention programs to combat obesity and its complications, so that survival is not accompanied by an increased burden of comorbidities.

New insights into causation of childhood 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 [25 26]. Hypertrophy and hyperplasia of the adipose tissue is characterized by local tissue hypoxia and activation of the inflammatory response, with secretion of inflammatory molecules called cytokines leading to local inflammation [27].

A major source of inflammatory cytokines in obese adipose tissue is an immune cell, the macrophage [25 26 28], 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 [29-35].

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 skeletal muscle and liver.

Fatty acids exert their effects in two different ways. They can bind to immune cell receptors called Toll-Like Receptors [TLR] that include TLR2 and TLR4 and initiate signaling through the receptor and its signaling pathway. These receptors are present on the cell surface of macrophages and metabolic cells [36 37]. Alternatively, fatty acids may be transported intracellularly and metabolized to generate lipid intermediates including ceramides and diacylglycerol.

Both inflammatory cytokines and fatty acids and their metabolites collaborate to trigger the activation of intracellular inflammatory pathways including c-Jun N-terminal kinase [JNK], Protein Kinase C [PKC], and Inhibitor of nuclear factor-kappa B kinase-β [IKKβ].

The activation of these pathways will stimulate further cytokine production, leading to inhibition of insulin signaling and insulin resistance in metabolic organs [5 38]. With insulin resistance, a compensatory increase in endogenous

insulin production ensues, leading to hyperinsulinemia. When pancreatic insulin production fails to keep up with demand, type 2 diabetes develops.

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 occur very shortly after exposure. If innate immune responses are not sufficient to combat the threat, then adaptive immunity is activated.

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.

Some of the innate immune system components include immune cells like monocytes and neutrophils, and receptors including TLRs noted above. The monocytes are attracted to metabolic organs in obesity, 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 liver [29 39 40].

The anti-inflammatory or ‘M2’ macrophages are resident macrophages exist under physiological conditions and help with tissue homeostasis and remodeling, and reduce adipose tissue inflammation in obesity [41].

Another source of M2 macrophages is monocytes recruited during weight loss, which helps with processing of fatty acids in adipose tissue during this phase; the numbers of these cells drop once weight loss is achieved [42]. The loss of anti-inflammatory actions of M2 macrophages and augmented inflammatory responses by M1 macrophages is considered a central driver of the adverse effects of inflammation in obesity [43].

Animal and adult human studies clearly document the presence of inflammation and the activation of innate immunity in obesity [44-46]. On the other hand, little systematic inquiry has been done to elucidate the immunometabolic interactions in childhood obesity [47-58], and how this shapes the landscape in children for future metabolic risk. This is important, as some of the mechanisms that may be hard-wired in adults may still be amenable to modification in children, and we know nothing about these mechanisms and their potential reversibility in children, and this is also the case for those children who have survived brain tumors.

In the pediatric literature, the evidence for innate immune system activation in obese children is limited. One study reported that obese children had a higher number of circulating monocytes compared to lean children [59]. Few papers have shown evidence of elevation of inflammatory markers in obesity [60-62]. Virtually nothing is known about the immune system activation in obese children, and molecules and pathways involved in mediating this activation.

In our cohort, we found that obesity rates in survivors of childhood brain tumors are around 44%, almost two-fold higher than the general population [unpublished].

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 DNA called messenger RNA (mRNA). The latter is then used to synthesize 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 CpG dinucleotide area in the gene promoter. The methylation status of a gene is important in determining its transcription, and new understanding indicates that spatial location of methylation is important in activating or silencing gene transcription [63]. We have no knowledge of cytokine gene methylation role in inflammation in obese survivors of childhood brain tumors or their obese controls with no history of tumors.

The aim of this study is to determine if obese survivors of childhood brain tumors have more inflammation than lean survivors, and lean and obese children with no history of tumors.

In addition, we wish to systematically evaluate the potential mechanisms involved in the occurrence of inflammation in these groups.

Hypotheses

Primary hypothesis:

Obese survivors of childhood brain tumors have enhanced inflammation when compared to lean survivors and to lean and obese children with no history of tumors. This inflammatory response is due to upregulation of monocyte TLR and inflammatory cytokine gene expression due to altered gene methylation patterns.

Secondary hypothesis: The inflammatory response seen in obese survivors of childhood brain tumors when compared to lean survivors and to lean and obese children with no history of tumors is mediated via individual and lifestyle factors.

Objectives (Table 1)

Primary objectives:

1. To determine inflammatory cytokine levels in obese survivors of childhood brain tumors, lean survivors and compare those levels to lean and obese children with no history of tumors
2. To quantify monocyte TLR and inflammatory cytokine gene expression in obese survivors of childhood brain tumors, lean survivors and lean and obese children with no history of tumors.
3. Determine Methylation patterns of monocyte TLR pathway and inflammatory cytokine genes in obese survivors of childhood brain tumors, lean survivors and lean and obese children with no history of tumors.

Secondary objectives:

To Determine the relation between diet, physical fitness, sleep, stress, and built environment and cytokine levels, gene expression and DNA methylation patterns of TLR and cytokine genes in obese survivors of childhood brain tumors compared to lean survivors and lean and obese children with no history of tumors.

Study methods & 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 two-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 5-18 years old, and who are lean (BMI below 85th percentile for age and gender) or overweight/obese (BMI above 85th percentile for age and gender). In addition, potential participants should be infection-free and have no history of autoimmune diseases, high dose steroid or immunosuppressive therapy for 15 days prior to participation. In addition, for those with history of brain tumors, they should have completed therapy for at least 6 months prior to enrollment. Exclusion criteria include history of smoking, active infection, or inability or refusal to provide consent.

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 7-15 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 followed by phlebotomy station for blood samples collection, and the provision of containers to obtain saliva and urine samples. Samples will be processed within 60 minutes of collection, and we will promptly anonimize the samples using unique identifying numbers to protect confidentiality.

Measurements

The participant height is measured closest to 0.1 cm using a stadiometer, weight to closest 0.1kg using weighing scale, and Body Mass Index (BMI) in kg/m^2 calculated from height and weight. We will measure waist circumference and hip circumference using a spring-loaded measuring tape closest to 0.1cm. Sitting right arm systolic and diastolic blood pressure (BP) and pulse rate is measured twice using automated BP and heart rate monitor. Body fat percent is measured using Tanita body fat monitor for children, and grip strength is tested using a Dynamometer.

Questionnaires

We will collect sociodemographic data including age, gender, school grade or job description, parental education, religion, ethnicity, birth history, family history, feeding, and vaccinations from all participants. In addition, tumor type, treatment protocol, complications of tumor or its therapy, history of medical or surgical problems will be collected for survivors of childhood brain tumors. 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 staging, we will use pictorial depiction of puberty for breasts in girls 8 years or older, and for the external genitalia for boys 9 years and older.

Regarding dietary information, we will use a food frequency questionnaire along with questions about sugary drinks and eating behaviors to collect this data. We will measure physical activity using the HAES questionnaire [64].

We will measure sleep duration and quality using a standardized sleep questionnaire [65], and will evaluate built environment using a validated tool [66]. We will also enquire about mental health issues using a questionnaire reporting mood disorders [67].

Blood sampling

Certified phlebotomists in the Hospital or trained health care professionals will take fasting blood samples. These samples include serum, plasma, complete blood counts, and samples to isolate white blood cells (leukocytes). The total volume of blood needed is around 20 milliliters. 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

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 saliva collection kit. Samples are stored at room temperature until further analysis.

Experimental work:

Determination of circulating & urinary cytokine levels

The study will have four arms including obese survivors of childhood brain tumors (OBT), lean survivors of childhood brain tumors (LBT), obese children with no history of tumors (ONB), and lean children with no history of tumors (LNB).

We will quantify cytokine concentrations in the serum and urine using the Bioplex ELISA kits (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 manufacturer's instructions. Cells will be sorted based on monocyte markers CD14 and CD16.

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

TLR pathway gene expression profiling looking at 84 genes involved in TLR expression and signaling pathway will be done using Human Toll-Like Receptor Signaling Pathway PCR Array (SABiosciences) as per manufacturer's instructions.

Determination of Methylation patterns of monocyte TLR pathway and cytokine genes

We will isolate genomic DNA from monocytes using DNAeasy Mini kit (Qiagen) as per manufacturer's instructions. The DNA will then be processed for qRT-PCR reaction using SYBR Green reaction master mix to measure methylation patterns for 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, Orthopedics and Cardiology services. The Neurooncology program at our institution cares for 270 survivors of childhood brain tumors and the program reviews patients annually or more frequently depending of the tumor type and time from completion of therapy, with clinics serving 8 patients per

week. The Orthopedics clinic serves 70-80 patients per week and the Cardiology service performs assessments of 24 patients per week. Assuming 50% recruitment rate based on fulfillment of inclusion criteria and interest in participation, our goal is to recruit 150 lean and obese brain tumor survivors and 150 lean and obese children with no history of tumors, at a rate of three patients per week over a two-year period.

Based on these figures, we estimate to have 99.7% power to reject the null hypothesis with alpha set at 0.05, and the difference in population means is 12 pg/ml, and a difference within a group of 21.9 pg/ml. The latter figure is set based on a previous study with one cytokine to determine the presence of inflammation in a sample of lean and obese children (unpublished). Importantly, to obtain 80% power, we will need 29 participants in each of the groups, and these calculations are done using Power and Sample Size Calculations software version 3.0.43 [68]. Our overall recruitment target is consistent with reported enrollment rates of children with cancer in clinical trials, with rates ranging from 70% participation rate in the 0-14 year old group [69] and dropping to 24% in 15-19 year old category, and other earlier studies demonstrating even lower rates of recruitment in the latter age group [70-72].

There is conflicting evidence regarding the participation of ethnic minorities in pediatric cancer studies, with some showing appropriate representation while others documenting underrepresentation [73-75]. 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 summarized using descriptive summary measures expressed as mean (standard deviation) or median (minimum-maximum) for continuous variables and number (percent) for categorical variables. In addition, we will test for differences in sociodemographic and baseline clinical characteristics between groups using chi-square tests for categorical variables and one-way analysis of variance (ANOVA) 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 95% confidence interval and associated p-values. Statistical significance will be set at alpha = 0.05 adjusted using Bonferroni approach for multiple analyses. We will examine the residuals to assess model assumptions. All analyses will be performed using SAS 9.2 (Cary, NC) or SPSS (Chicago, IL) statistical software.

Discussion:

In this study, we will investigate the mechanisms of inflammation in obese survivors of childhood brain tumors and compare them to lean survivors and lean and obese children with no history of tumors.

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 pediatric age group and express inflammatory markers, then interventions may have to be more aggressive including life style intervention, nutraceutical and pharmacological treatments to address these mechanisms.

Understanding 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 randomized controlled trial using nutraceutical of pharmacological therapy with or without life style intervention to identify the most effective intervention(s) in ameliorating inflammation, and if this occur via modulation of 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 the lean survivors or lean and obese children in the non-tumor 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 tumors, and 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 co-morbid associations.

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Authors' contribution: MCS conceived the study idea and generated the hypotheses. MCS, LT, RD, SB, and KS finalized the study design, and LT and MCS completed the statistical analysis plans. MCS, RD, SB, and KS contributed to the definition of study cohorts, inclusion and exclusion criteria, recruitment plan, and study logistics including space and resource allocation. MCS wrote the manuscript draft and all authors contributed to the current version.

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Table 1: DECIDE study objectives and statistical analysis plan.

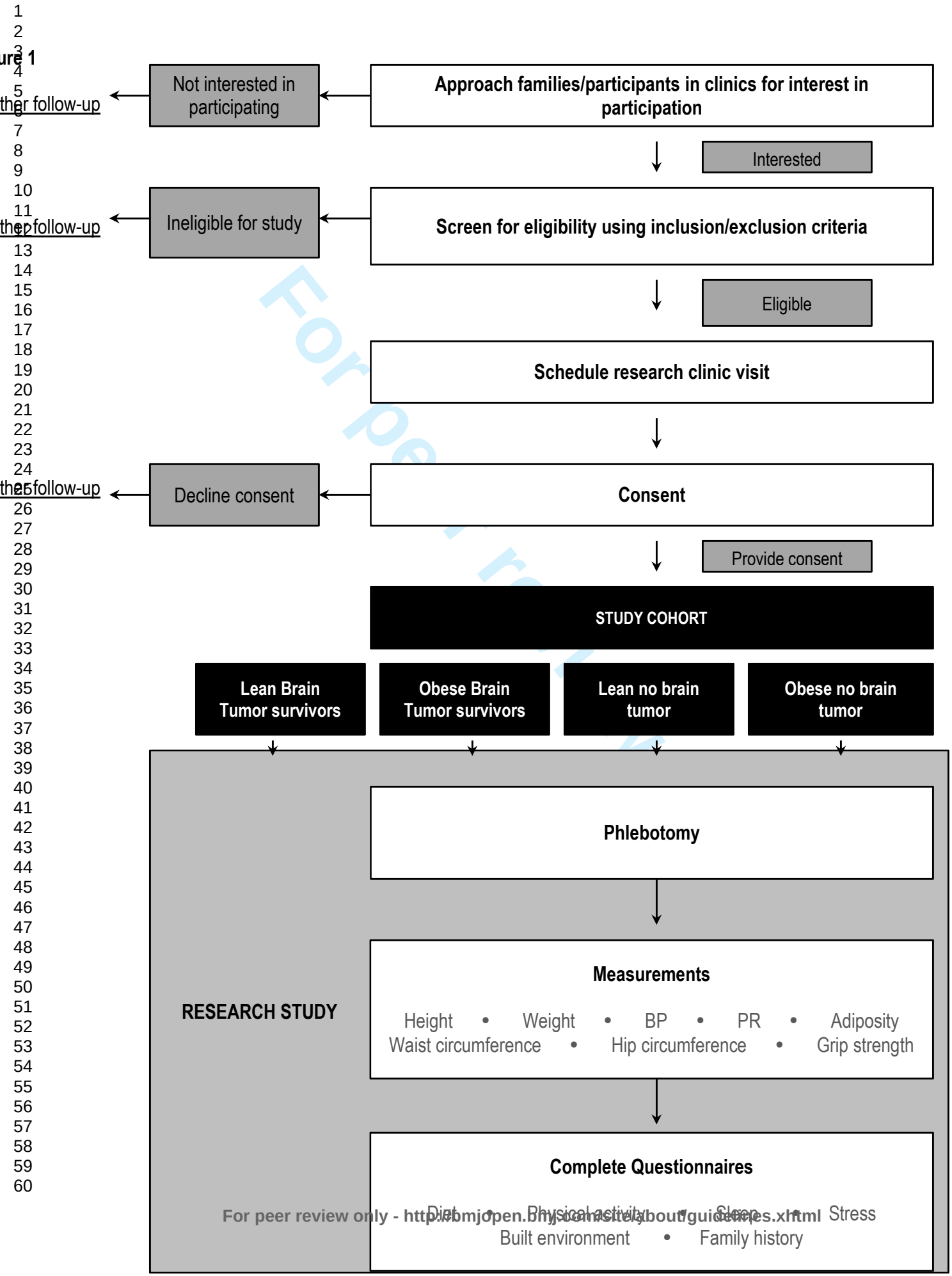
Figure 1: Flow chart of the DECIDE study.

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 enrollment. The participants will be stratified into the four arms of the study including obese childhood survivors of brain tumors (OBT), lean childhood survivors of brain tumors (LBT), obese children with no history of tumors (ONB), and lean children with no history of tumors (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.

Table 1: DECIDE study objectives and statistical analysis plans

Objectives	Outcome	Independent variables	Hypotheses	Statistical analysis
Primary				
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 tumors and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors	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 versus controls	Age Sex Ethnicity Puberty BMI Lipid levels HOMA-IR	Obese survivors of childhood brain tumors have higher inflammatory status and altered gene methylation patterns compared to lean survivors and non-cancer controls that predisposes them to Endometabolic risks	Regression

Objectives	Outcome	Independent variables	Hypotheses	Statistical analy
Secondary Determination of role of 1. Sleep 2. Stress 3. Diet 4. Adiposity 5. Fitness 6. Physical activity 7. Built environment That impacts circulating cytokine levels, gene expression and DNA methylation patterns of TLR and cytokine genes in obese survivors of childhood brain tumors and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors.	Understand the role of 1. Sleep 2. Stress 3. Diet 4. Adiposity 5. Fitness 6. Physical activity 7. Built environment On development of inflammation in obese survivors of childhood cancer and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors.	Age Sex Ethnicity Puberty BMI Lipid levels HOMA-IR	Obese survivors of childhood brain tumors have higher inflammatory status and altered gene methylation patterns, and this is mediated via individual and lifestyle factors	Descriptive analysis Regression



Article summary:

Article focus:

- The goal of DECIDE study is to determine if obese survivors of childhood brain tumors have more inflammation than lean survivors, and lean and obese children with no history of tumors.
- It will also evaluate the potential mechanisms involved in the occurrence of inflammation in these groups.

Key messages:

-This study will determine the inflammation status of obese survivors of childhood brain tumors and children with no history of cancer.

-This may allow the determination of preventative and therapeutic strategies to mitigate the risk of obesity and its comorbidities in this population.

Strengths & limitations of the study:

-The strength of this study is that it will systematically study the inflammatory response in childhood obesity in pediatric brain tumor 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 will be used if this is the case.

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 tumors

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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 tumors

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Abstract:

Introduction: 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 pediatric groups at risk of higher rates of obesity includes the survivors of childhood brain tumors.

The aim of this study is to evaluate the mechanisms that contribute to inflammation in obese survivors of childhood brain tumors.

Methods & analysis: This is a prospective cohort study. We will recruit lean and obese survivors of childhood brain tumors, and a control group composed of lean and obese children with no history of tumors. We will measure circulating and urinary cytokine levels and cytokine gene expression in monocytes. In addition, 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.

Sample size calculation showed that we need 29 participants per arm.

Ethics & 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 tumors and comparing them to lean survivors and lean and obese controls with no history of tumors, which may help identify therapeutic and preventative interventions to combat the rising tide of obesity.

Keywords: Childhood obesity, brain tumor, survivorship, Immunometabolism, inflammation, Toll-Like Receptors, cytokines

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 5 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, dyslipidemia 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 aging 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 tumors [16 17]. What is not clear so far is if childhood obesity increases the risk of tumors during childhood, and its potential effect on long-term metabolic outcomes in those who survive childhood cancer.

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

Survivors of childhood brain tumors have a higher risk of obesity

Brain tumors 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 lead 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 23 24-26 27 28]. The etiology of obesity in brain tumor survivors is polygenic and can be due to the tumor and its treatment interacting with the patient's genetic, epigenetic and environmental factors. In some tumors, including gliomas, the presence of obesity is a marker of poor prognosis [29].

Brain tumors can cause damage to the hypothalamic-pituitary region due to their location and size, with pressure and infiltration of surrounding structures. In addition, injury to hypothalamic-pituitary structures can be secondary to chemotherapy [30], or due to radiotherapy when structures are in the path of radiation beams, or when tumors are adherent to surrounding structures and being removed surgically. Damage to the ventromedial hypothalamus impairs satiety/hunger signaling by leptin, ghrelin and insulin, all of which have hypothalamic receptors leading to hyperphagia. In addition, hypothalamic damage slows basal metabolic rate and causes 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 preventing 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 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 e.g. watching TV [23 28 41]. Furthermore, some of the drugs used in these patients during and after their brain tumor treatment are obesogenic including steroids, antidepressants, antipsychotics and anti-epileptic medications [42].

In addition to obesity increasing the risks of metabolic disease, survivors of childhood cancer have 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 were elevated when compared to 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 [47 44]. Hypertrophy and hyperplasia of the adipose tissue is characterized 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 skeletal muscle and liver [54 55 56 57].

Fatty acids exert their effects in two different ways. They can bind to receptors present on surface of immune and metabolic cells called Toll-Like Receptors [TLRs] that include TLR2 and TLR4 and initiate signaling through the receptor and its signaling pathway [58 59]. Alternatively, fatty acids may be transported intracellularly and metabolized to generate lipid intermediates including ceramides and diacylglycerol [54 60].

Both inflammatory cytokines and fatty acids and their metabolites collaborate to trigger the activation of intracellular inflammatory pathways including Mitogen Activated Protein Kinases (MAPKs), Protein Kinase C [PKC], and Inhibitor of nuclear factor-kappa B kinase-β [IKKβ] [49].

The activation of these pathways will stimulate further cytokine production, leading to inhibition of insulin signaling and insulin resistance in metabolic organs [61]. With insulin resistance, a compensatory increase in endogenous insulin production ensues, leading to hyperinsulinemia. When pancreatic insulin production fails to keep up with demand, type 2 diabetes develops [62 63].

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 occur very shortly after exposure. If innate immune

responses are not sufficient to combat the threat, then adaptive immunity is activated [63].

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 [64 49 65].

Some of the innate immune system components include immune cells like monocytes and neutrophils, and receptors including TLRs noted above. In addition to the production of cytokines, the inflammatory response in obesity is associated with the production of molecules called chemokines that help direct leukocytes 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 liver [47 66 67].

The anti-inflammatory or 'M2' macrophages are resident macrophages exist under physiological conditions and help with tissue homeostasis and remodeling, and reduce adipose tissue inflammation in obesity [68].

Another source of M2 macrophages is monocytes recruited during weight loss, which helps with processing of fatty acids in adipose tissue during this phase; the numbers of these cells drop once weight loss is achieved [69]. The loss of anti-inflammatory actions of M2 macrophages and augmented inflammatory

responses by M1 macrophages is considered a central driver of the adverse effects of inflammation in obesity [70].

Animal and adult human studies clearly document the presence of inflammation and the activation of innate immunity in obesity [71-73]. On the other hand, little systematic inquiry has been done to elucidate the immunometabolic interactions in childhood obesity [74-76], 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 compared to lean children [77]. Few papers have shown evidence of elevation of inflammatory markers in obesity [78-81].

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, and 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 tumors.

Local versus systemic inflammation in cancer

Local chronic inflammation in cancer is a well-established paradigm. Cancer is initiated by exposure to stimuli that leads to DNA and other cellular changes. These changes do not harm the host until a promoter is encountered [82], 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. Tumor cells secrete a mix of cytokines and chemokines that contribute to local inflammation and immune cell attraction.

The immune cells that are present in tumors include monocytes, neutrophils, eosinophils, dendritic cells, lymphocytes and mast cells [82]. These cells are bathed in cytokines, nutrients, and growth factors that constitute the tumor microenvironment, and they in turn start secreting their own array of cytokines in response to their environment. Both tumor and immune cells contribute to the creation of the tumor microenvironment that regulates tumor cell growth, invasion, metastasis and metabolism. It also regulates the differentiation of macrophages that associate with tumors called tumor associated macrophages (TAM) [83].

TAM play a critical role in defining the tumor microenvironment and tumor progression as they play a dual role in killing neoplastic cells, but also secrete angiogenic and lymphangiogenic factors and cytokines that help proliferation of tumors. In addition, they secrete Interleukin-10, which ameliorates the function of cytotoxic T-Lymphocytes responsible for tumor killing. These cells have been detected in brain tumors and their role in local inflammation is evolving [84 85], and it is unclear if they play a role in systemic inflammation in these patients.

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

tumor treatment that alters local or systemic inflammation and metabolism is unknown.

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

Understanding the mechanisms of immunometabolic interactions in obesity in this group may allow the design of effective treatment and prevention programs 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 DNA called messenger RNA (mRNA). The latter is then used to synthesize 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 CpG dinucleotide area in the gene promoter. The methylation status of a gene is important in determining its transcription, and new understanding indicates that spatial location of methylation is important in activating or silencing gene transcription [86]. We have no knowledge of cytokine gene methylation role in

inflammation in obese survivors of childhood brain tumors or their obese controls with no history of tumors.

Because obesity and cancer are inflammatory states, and as survivors of childhood brain tumors have multiple factors that contribute to a higher risk of obesity, the aim of this study is to determine if obese survivors of childhood brain tumors have enhanced inflammation when compared to lean survivors, and lean and obese children with no history of tumors.

Hypotheses

Primary hypothesis:

Obese survivors of childhood brain tumors have enhanced inflammation when compared to lean survivors and to lean and obese children with no history of tumors.

Secondary hypothesis: The inflammatory response seen in obese survivors of childhood brain tumors when compared to lean survivors and to lean and obese children with no history of tumors is mediated via individual and lifestyle factors**.**

Objectives

Primary objectives (Table 1):

1. To determine inflammatory cytokine levels in obese survivors of childhood brain tumors, lean survivors and compare those levels to lean and obese children with no history of tumors

2. To quantify monocyte TLR and inflammatory cytokine gene expression in obese survivors of childhood brain tumors, lean survivors and lean and obese children with no history of tumors.
3. Determine Methylation patterns of monocyte TLR pathway and inflammatory cytokine genes in obese survivors of childhood brain tumors, lean survivors and lean and obese children with no history of tumors.

Secondary objectives (Table 2):

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 obese survivors of childhood brain tumors compared to lean survivors and lean and obese children with no history of tumors.

Study methods & 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 two-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 (BMI below 85th percentile for age and gender), overweight (BMI between 85th 95th percentile for age and gender) and obese (BMI above 95th percentile 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/d), or inhaled steroids that are above the standard doses recommended for asthma therapy for 15 days prior to participation. In addition, for those with history of brain tumors, they should have completed therapy for at least 6 months prior to enrollment. Exclusion criteria include 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 7-15 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 followed by phlebotomy station for blood samples collection, and the provision of

containers to obtain saliva and urine samples. Samples will be processed within 60 minutes of collection, and we will promptly anonimize the samples using unique identifying numbers to protect confidentiality.

Measurements

The participant height is measured closest to 0.1 cm using a stadiometer, weight to closest 0.1kg using weighing scale, and Body Mass Index (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 0.1cm. Sitting right arm systolic and diastolic blood pressure (BP) and pulse rate is measured twice using automated BP and heart rate monitor. Total adiposity is measured by quantifying body fat percentage using Tanita body fat monitor for children (Tanita Corporation, IL, USA), and muscle strength is 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, tumor type, tumor location, treatment protocol, complications of tumor or its therapy, history of medical or surgical problems, and current medications including vitamin supplements will be collected for survivors of childhood brain tumors. 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[87], 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, and has recently been updated [88 89]. This is a questionnaire developed in a US pediatric cohort, and includes questions about food intake based on average portion sizes of different dietary constituents. In addition, questions about sugary drinks and eating behaviors are also collected. The number of servings per day will be calculated from the questionnaire by multiplying the frequency of consumption by portion size [90].

Physical activity. We will measure physical activity using the Habitual Activity Estimation Scale (HAES) questionnaire [91], which is used to measure physical activity in the pediatric 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 pediatric populations and those with chronic disease and correlates well with accelerometer data, and has high test-retest reliability [92]. Furthermore, we will also document motor disabilities while conducting these assessments.

Sleep. We will measure sleep duration and quality using a standardized pediatric sleep questionnaire [93], 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[93].

Stress. We will also enquire about mental health issues using a questionnaire reporting mood disorders [94]. We will screen for depressive symptoms using the Center for Epidemiological Studies Depression Scale for Children (CES-DC), 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 [94].

Built environment. We will evaluate built environment using the Neighborhood Environment Walkability Scale, which is a validated tool that measures factors related to neighborhood design, access, and safety [95]. These scores can then be correlated to physical activity levels and metabolic parameters.

Blood sampling

Certified phlebotomists in the Hospital or trained health care professionals will take fasting blood samples. These samples include serum, plasma, complete blood counts, and samples to isolate white blood cells (leukocytes). The total volume of blood needed is around 20 milliliters. 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 ℃ until further analysis.

Urine sampling

Participants will provide a urine sample in 90 ml plastic containers, and aliquots will be frozen at -80 ℃ 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 & urinary cytokine levels

The study will have four arms including obese survivors of childhood brain tumors (OBT), lean survivors of childhood brain tumors (LBT), obese children with no history of tumors (ONB), and lean children with no history of tumors (LNB).

We will quantify cytokine concentrations in the serum and urine using the Bioplex ELISA kits (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 manufacturer's instructions. Cells will be sorted based on monocyte markers CD14 and CD16.

We will isolate RNA using RNAeasy minikit (Qiagen). Quantification and determination of purity will be done using nanospectrophotometer. SuperScript III

reverse transcriptase kit (Invitrogen) will be used to generate cDNA as per manufacturer's instructions. We will use TaqMan probes (Applied Biosystems) to measure gene expression status of cytokines (TNFα, IL-1β, IL-6, CCL-2, IL-8, IL-10, IL-18, MIP-1α) by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

TLR pathway gene expression profiling looking at 84 genes involved in TLR expression and signaling pathway will be done using Human Toll-Like Receptor Signaling Pathway PCR Array (SABiosciences) as per manufacturer's instructions.

Determination of Methylation patterns of monocyte TLR pathway and cytokine genes

We will isolate genomic DNA from monocytes using DNAeasy Mini kit (Qiagen) as per manufacturer's instructions. The DNA will then be processed for qRT-PCR reaction using SYBR Green reaction master mix to measure methylation patterns for 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, Orthopedics and Cardiology services. The Neurooncology program at our institution cares for 270 survivors of childhood brain tumors and the program reviews patients annually or more frequently depending of the tumor type and time from completion of therapy, with clinics serving 8 patients per week. The Orthopedics clinic serves 70-80 patients per week and the Cardiology service performs assessments of 24 patients per week. Assuming 50%

recruitment rate based on fulfillment of inclusion criteria and interest in participation, our goal is to recruit 150 lean and obese brain tumor survivors and 150 lean and obese children with no history of tumors, at a rate of three patients per week over a two-year period.

Based on these figures, we estimate to have 99.7% power to reject the null hypothesis with alpha set at 0.05, and the difference in population means is 12 pg/ml, and a difference within a group of 21.9 pg/ml. The latter figure is set based on a previous study with one cytokine to determine the presence of inflammation in a sample of lean and obese children (unpublished). Importantly, to obtain 80% power, we will need 29 participants in each of the groups, and these calculations are done using Power and Sample Size Calculations software version 3.0.43 [96]. Our overall recruitment target is consistent with reported enrollment rates of children with cancer in clinical trials, with rates ranging from 70% participation rate in the 0-14 year old group [97] and dropping to 24% in 15-19 year old category, and other earlier studies demonstrating even lower rates of recruitment in the latter age group [98-100].

There is conflicting evidence regarding the participation of ethnic minorities in pediatric cancer studies, with some showing appropriate representation while others documenting underrepresentation [101-103]. 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 summarized using descriptive summary measures expressed as mean (standard deviation) or median (minimum-maximum) for continuous variables and number (percent) for categorical variables. In addition, we will test for differences in sociodemographic and baseline clinical characteristics between groups using chi-square tests for categorical variables and one-way analysis of variance (ANOVA) 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 95% confidence interval and associated p-values. Statistical significance will be set at alpha = 0.05 adjusted using Bonferroni approach for multiple analyses. We will examine the residuals to assess model assumptions. All analyses will be performed using SAS 9.2 (Cary, NC) or SPSS (Chicago, IL) statistical software.

The majority of tumors in our subjects are divided into three groups including gliomas, medulloblastomas, and ependymomas. Other less common tumors include PNET, and a mix of germ cell tumors, germinoma, atypical Neurocytoma, ATRT, and other rare tumors. 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 tumor 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 obese survivors of childhood brain tumors and compare them to lean survivors and lean and obese children with no history of tumors.

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 pediatric age group and express inflammatory markers, then interventions may have to be more aggressive including life style intervention, nutraceutical and pharmacological treatments to address these mechanisms.

Understanding 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 randomized controlled trial using nutraceutical of pharmacological therapy with or without life style intervention to

identify the most effective intervention(s) in ameliorating inflammation, and if this occur via modulation of 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 the lean survivors or lean and obese children in the non-tumor 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 tumors, and 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 co-morbid associations.

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Authors' contribution: MCS conceived the study idea and generated the hypotheses. MCS, LT, RFD, SB, and KS finalized the study design, and MCS and LT completed the statistical analysis plans. MCS, RFD, SB, and KS contributed to the definition of study cohorts, inclusion and exclusion criteria, recruitment plan, and study logistics including space and resource allocation. MCS wrote the manuscript draft and all authors reviewed the current version.

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Competing interests: The authors declare that they do not have competing interests

Table 1: CanDECIDE study primary objectives and statistical analysis plan.

Table 2: CanDECIDE study secondary objectives and statistical analysis plan.

Figure 1: Flow chart of the 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 enrollment. The participants will be stratified into the four arms of the study including obese childhood survivors of brain tumors (OBT), lean childhood survivors of brain tumors (LBT), obese children with no history of tumors (ONB), and lean children with no history of tumors (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.

Table 1: CanDECIDE study primary objectives and statistical analysis plans

Objectives	Outcome	Independent variables	Hypotheses	Statistical analys
Primary				
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 tumors and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors	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 versus controls	1. Age 2. Sex 3. Ethnicity 4. Puberty 5. Tumor type 6. Tumor location 7. Tumor treatment 8. Hormonal deficiencies 9. BMI 10. Lipid levels 11. HOMA-IR	Obese survivors of childhood brain tumors have higher inflammatory status and altered gene methylation patterns compared to lean survivors and non-cancer controls that predisposes them to Endometabolic risks	Regression

Table 2: CanDECIDE study secondary objectives and statistical analysis plans

Objectives	Outcome	Independent variables	Hypotheses	Statistical analy
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 tumors and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors.	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 to non-obese tumor survivors and lean and obese subjects with no history of tumors.	1. Age 2. Sex 3. Ethnicity 4. Puberty 5. Physical activity 6. Tumor type 7. Tumor location 8. Tumor treatment 9. Hormonal deficiencies 10. BMI 11. Lipid levels 12. HOMA-IR	Obese survivors of childhood brain tumors have higher inflammatory status and altered gene methylation patterns, and this is mediated via individual and lifestyle factors	Descriptive analysis Regression

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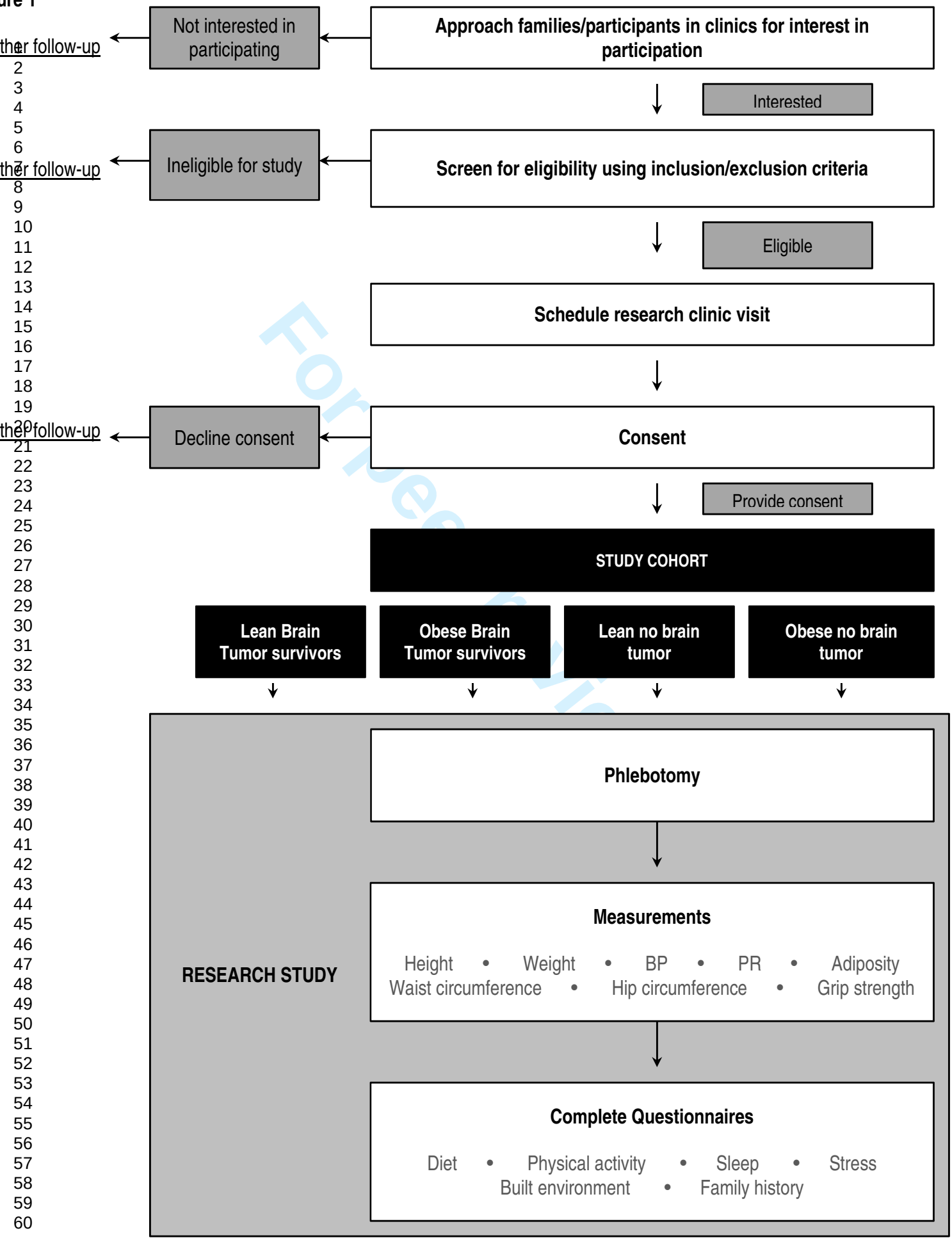

Article summary:

Article focus:

- The goal of CanDECIDE study is to determine if obese survivors of childhood brain tumors have more inflammation than lean survivors, and lean and obese children with no history of tumors.
- 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 of obese survivors of childhood brain tumors and children with no history of cancer.

-This may allow the determination of preventative and therapeutic strategies to mitigate the risk of obesity and its comorbidities in this population.

Strengths & limitations of the study:

-The strength of this study is that it will systematically study the inflammatory response in childhood obesity in pediatric brain tumor 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.

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 tumors

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Word count: 4,372 words

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Abstract:

Introduction: 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 pediatric groups at risk of higher rates of obesity includes the survivors of childhood brain tumors.

The aim of this cohort study is to evaluate the mechanisms that contribute to inflammation in obese survivors of childhood brain tumors.

Methods & analysis: This is a prospective cohort study. We will recruit lean and obese survivors of childhood brain tumors, and a control group composed of lean and obese children with no history of tumors. We will ~~The groups will be evaluated for their inflammatory profile including the~~ measure~~ment of~~ circulating and urinary cytokine levels and cytokine gene expression in monocytes. In addition, 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.

Sample size calculation showed that we need 29 participants per arm.

Ethics & 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 tumors and comparing them to lean survivors and lean and obese controls with no history of tumors, which may help identify therapeutic and preventative interventions to combat the rising tide of obesity.

Keywords: Obesity, brain tumor survivorship, Immunometabolism, inflammation, Toll-Like Receptors, cytokines

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 5 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, dyslipidemia and ~~chronic diseases like~~ type 2 diabetes during childhood. In addition, obese children are likely to become obese adults [10-13], ~~and this~~ increasing ~~es~~ their risk of type 2 diabetes and cardiovascular disease [2 14]. ~~These~~ These children are developing diseases of adults at an ever younger age, defining obesity as a state of premature aging that impacts the longevity and ~~the~~ quality of life ~~and life span~~ of a generation that ~~of children who~~ will live with obesity and its comorbidities for decades, as they are likely to live longer.

In addition to the above cardiometabolic complications, adult o~~Evidence links o~~besity is associated with increased risk of certain cancers~~tumor~~ s ~~and may also~~ impact ~~tumor~~ treatment outcomes ~~in adults~~ [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. ~~Recent~~ ~~indications are linking~~ childhood obesity is associated with ~~with~~ increase~~d~~ ~~d~~ risk of adult colon and urothelial tumors [16 17]. What is not clear so far is

if ~~the influence of~~ childhood obesity ~~,~~ increases the risk of ~~on the probability of~~ tumors during childhood, and its potential effect on long-term metabolic outcomes in those who survive childhood cancer.

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

Survivors of childhood brain tumors have a higher risk of obesity

Brain tumors 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 lead to a significant reduction in mortality [19 20]. As survival rates improved, it became ~~is~~ apparent that these patients have higher premature mortality [21] and morbidity rates, and one such morbidity is obesity [22 23].

~~However, survivors face a number of morbidities that impact their overall health [24-26 27 28]. As survival rates improve, it is apparent that these patients have higher premature mortality [27] and morbidity rates, and one such morbidity is obesity [28 29].~~

The etiology of obesity in brain tumor survivors is polygenic and can be due to the tumor and its treatment interacting with the patient's genetic, epigenetic and environmental factors. In some tumors, including gliomas, the presence of obesity is a marker of poor prognosis [29].

Brain tumors can cause damage to the hypothalamic-pituitary region due to their location and size, with pressure and infiltration of surrounding structures. In

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addition, injury to hypothalamic-pituitary structures can be secondary to chemotherapy [30], or due to radiotherapy when structures are in the path of radiation beams, or when tumors are adherent to surrounding structures and being removed surgically. Damage to the ventromedial hypothalamus impairs satiety/hunger signaling by leptin, ghrelin and insulin, all of which have hypothalamic receptors leading to hyperphagia. In addition, hypothalamic damage slows basal metabolic rate and causes 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 preventing 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 37], or cardiac disease due to the effects of chemotherapy or radiation on the heartdamage [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

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way the child is perceived to have different exercise tolerance and ability to handle physical activity and is allowed to develop sedentary habits e.g. watching TV [23 28 41]. Furthermore, some of the drugs used in these patients during and after their brain tumor treatment are obesogenic including steroids, antidepressants, antipsychotics and anti-epileptic medications [42].

In addition to obesity increasing the risks of metabolic disease, survivors of childhood cancer have increased 30-year risk profile for myocardial infarction, stroke, and coronary death whether or not they received cardiotoxic therapy ~~were treated with surgery, radiotherapy or chemotherapy~~ [43]. Importantly, ~~these patients have~~ non-high density lipoprotein, ~~abnormal lipid profiles, elevated~~ insulin levels, and high C-reactive protein were elevated ~~and greater concentrations of the inflammatory marker C-Reactive Protein (CRP)~~ when compared to non-cancer controls. This indicates that cancer itself is associated with ~~systemic inflammation, and this plays a role in the~~ the pathogenesis of adverse cardiometabolic outcomes in these patients and obesity may add to this risk [43].

~~There are no studies so far that have interrogated the activation of immune system and inflammatory pathways in survivors, and CRP is not a good marker on its own to characterize the inflammatory responses or to inform the mechanisms behind its development.~~

~~Understanding the fundamental mechanisms of obesity development in this group will allow the design of effective treatment and prevention programs to~~

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~~combat obesity and its complications, so that survival is not accompanied by an increased burden of comorbidities.~~

Newew insights into causation of _ obesity: immune system activation and inflammation

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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[47 44]. Hypertrophy and hyperplasia of the adipose tissue is characterized 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 skeletal muscle and liver [54 55 56 57].

Fatty acids exert their effects in two different ways. They can bind to receptors present on surface of immune and metabolic cells called Toll-Like Receptors [TLRs] that include TLR2 and TLR4 and initiate signaling through the receptor

and its signaling pathway. ~~These receptors are present on the cell surface of macrophages and metabolic cells~~ [58 59]. Alternatively, fatty acids may be transported intracellularly and metabolized to generate lipid intermediates including ceramides and diacylglycerol [54 60].

Both inflammatory cytokines and fatty acids and their metabolites collaborate to trigger the activation of intracellular inflammatory pathways including Mitogen Activated Protein Kinases (MAPKs), Protein Kinase C [PKC], and Inhibitor of nuclear factor-kappa B kinase-β [IKKβ] [49~~55~~].

The activation of these pathways will stimulate further cytokine production, leading to inhibition of insulin signaling and insulin resistance in metabolic organs [61~~55 7 61~~]. With insulin resistance, a compensatory increase in endogenous insulin production ensues, leading to hyperinsulinemia. When pancreatic insulin production fails to keep up with demand, type 2 diabetes develops [62 63].

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 occur very shortly after exposure. If innate immune responses are not sufficient to combat the threat, then adaptive immunity is activated [63].

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 [64 49 65].

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Some of the innate immune system components include immune cells like monocytes and neutrophils, and receptors including TLRs noted above. In addition to the production of cytokines, the inflammatory response in obesity is associated with the production of molecules called chemokines that help direct leukocytes into metabolic organs. Through the actions of chemokines, circulating ~~The~~ monocytes are attracted to metabolic organ~~ss in obesity~~, 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 liver [47 66 67].

The anti-inflammatory or 'M2' macrophages are resident macrophages exist under physiological conditions and help with tissue homeostasis and remodeling, and reduce adipose tissue inflammation in obesity [68].

Another source of M2 macrophages is monocytes recruited during weight loss, which helps with processing of fatty acids in adipose tissue during this phase; the numbers of these cells drop once weight loss is achieved [69]. The loss of anti-inflammatory actions of M2 macrophages and augmented inflammatory responses by M1 macrophages is considered a central driver of the adverse effects of inflammation in obesity [70].

Animal and adult human studies clearly document the presence of inflammation and the activation of innate immunity in obesity [71-73]. On the other hand, little systematic inquiry has been done to elucidate the immunometabolic interactions in childhood obesity [74-76], 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 compared to lean children[77]. Few papers have shown evidence of elevation of inflammatory markers in obesity[78-81]

ItThis 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, and 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 tumors.

Local versus systemic inflammation in cancer

~~In the pediatric literature, the evidence for innate immune system activation in obese children is limited. One study reported that obese children had a higher number of circulating monocytes compared to lean children[88]. Few papers have shown evidence of elevation of inflammatory markers in obesity[89-92]. Virtually nothing is known about the immune system activation in obese children, and molecules and pathways involved in mediating this activation.~~

Local chronic inflammation in cancer is a well-established paradigm. Cancer is initiated by exposure to stimuli that leads to DNA and other cellular changes. These changes do not harm the host until a promoter is encountered[82], 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.

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Tumor cells secrete a mix of cytokines and chemokines that contribute to local inflammation and immune cell attraction.

The immune cells that are present in tumors include monocytes, neutrophils, eosinophils, dendritic cells, lymphocytes and mast cells [82]. These cells are bathed in cytokines, nutrients, and growth factors that constitute the tumor microenvironment, and they in turn start secreting their own array of cytokines in response to their environment. Both tumor and immune cells contribute to the creation of the tumor microenvironment that regulates tumor cell growth, invasion, metastasis and metabolism. It also regulates the differentiation of macrophages that associate with tumors called tumor associated macrophages (TAM) [83].

TAM play a critical role in defining the tumor microenvironment and tumor progression as they play a dual role in killing neoplastic cells, but also secrete angiogenic and lymphangiogenic factors and cytokines that help proliferation of tumors. In addition, they secrete Interleukin-10, which ameliorates the function of cytotoxic T-Lymphocytes responsible for tumor killing. These cells have been detected in brain tumors and their role in local inflammation is evolving [84 85], and it is unclear if they play a role in systemic inflammation in these patients.

The presence of systemic inflammation in pediatric brain tumors is not well studied. In a recent report, survivors of childhood tumors, including a small group of brain tumor survivors, were demonstrated to have hyperinsulinism, dyslipidemia and elevated CRP. This was independent of treatment status, which argues for a direct role of the tumor itself or perhaps its immune cell

complement in affecting systemic metabolism and inflammation. Whether this is the case, of indeed if these cells leave an inflammation signature even after tumor treatment that alters local or systemic inflammation and metabolism is unknown.

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

Understanding the mechanisms of immunometabolic interactions in obesity in this group may allow the design of effective treatment and prevention programs 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 DNA called messenger RNA (mRNA). The latter is then used to synthesize 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 CpG dinucleotide area in the gene promoter. The methylation status of a gene is important in determining its transcription, and new understanding indicates that

spatial location of methylation is important in activating or silencing gene transcription [86]. We have no knowledge of cytokine gene methylation role in inflammation in obese survivors of childhood brain tumors or their obese controls with no history of tumors.

Because obesity and cancer are inflammatory states, and as survivors of childhood brain tumors have multiple factors that contribute to a higher risk of obesity,,the aim of this study is to determine if obese survivors of childhood brain tumors have enhanced inflammation when compared to lean survivors,and lean and obese children with no history of tumors. ~~In addition, we wish to systematically evaluate the potential mechanisms involved in the occurrence of inflammation in these groups.~~

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Hypotheses

Primary hypothesis:

Obese survivors of childhood brain tumors have enhanced inflammation when compared to lean survivors and to lean and obese children with no history of tumors. ~~This inflammatory response is due to upregulation of monocyte TLR and inflammatory cytokine gene expression due to altered gene methylation patterns.~~

Secondary hypothesis: The inflammatory response seen in obese survivors of childhood brain tumors when compared to lean survivors and to lean and obese children with no history of tumors is mediated via individual and lifestyle factors.

Objectives ~~(Table 1)~~

Primary objectives (Table 1):

1. To determine inflammatory cytokine levels in obese survivors of childhood brain tumors, lean survivors and compare those levels to lean and obese children with no history of tumors
2. To quantify monocyte TLR and inflammatory cytokine gene expression in obese survivors of childhood brain tumors, lean survivors and lean and obese children with no history of tumors.
3. Determine Methylation patterns of monocyte TLR pathway and inflammatory cytokine genes in obese survivors of childhood brain tumors, lean survivors and lean and obese children with no history of tumors.

Secondary objectives (Table 2):

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 obese survivors of childhood brain tumors compared to lean survivors and lean and obese children with no history of tumors.

Study methods & 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 two-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 (BMI below 85th percentile for age and gender), overweight (BMI between 85th

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95th percentile for age and gender) and obese (BMI above 95th percentile 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/d), or inhaled steroids that are above the standard doses recommended for asthma therapy for 15 days prior to participation. In addition, for those with history of brain tumors, they should have completed therapy for at least 6 months prior to enrollment. Exclusion criteria include 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 7-15 years of age, and separate consent form for genetic (DNA) testing.

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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 followed by phlebotomy station for blood samples collection, and the provision of containers to obtain saliva and urine samples. Samples will be processed within 60 minutes of collection, and we will promptly anonimize the samples using unique identifying numbers to protect confidentiality.

Measurements

The participant height is measured closest to 0.1 cm using a stadiometer, weight to closest 0.1kg using weighing scale, and Body Mass Index (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 0.1cm. Sitting right arm systolic and diastolic blood pressure (BP) and pulse rate is measured twice using automated BP and heart rate monitor. Total adiposity is measured by quantifying body fat percentage using Tanita body fat monitor for children (Tanita Corporation, IL, USA), and muscle strength is 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, tumor type, tumor location, treatment protocol, complications of tumor or its therapy, history of medical or surgical problems, and

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current medications including vitamin supplements will be collected for survivors of childhood brain tumors. 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[87], 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, and has recently been updated [88,89]. This is a questionnaire developed in a US pediatric cohort, and includes questions about food intake based on average portion sizes of different dietary constituents. In addition, questions about sugary drinks and eating behaviors are also collected. The number of servings per day will be calculated from the questionnaire by multiplying the frequency of consumption by portion size [90].

Physical activity. We will measure physical activity using the Habitual Activity Estimation Scale (HAES) questionnaire [91], which is used to measure physical activity in the pediatric 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.

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HAES has been used in healthy pediatric populations and those with chronic disease and correlates well with accelerometer data, and has high test-retest reliability [92]. Furthermore, we will also document motor disabilities while conducting these assessments.

Sleep. We will measure sleep duration and quality using a standardized pediatric sleep questionnaire [93], 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[93].

Stress. We will also enquire about mental health issues using a questionnaire reporting mood disorders [94]. We will screen for depressive symptoms using the Center for Epidemiological Studies Depression Scale for Children (CES-DC), 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 [94].

Built environment. We will evaluate built environment using the Neighborhood Environment Walkability Scale, which is a validated tool that measures factors related to neighborhood design, access, and safety [95]. These scores can then be correlated to physical activity levels and metabolic parameters.

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Blood sampling

Certified phlebotomists in the Hospital or trained health care professionals will take fasting blood samples. These samples include serum, plasma, complete blood counts, and samples to isolate white blood cells (leukocytes). The total

volume of blood needed is around 20 milliliters. 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

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 & urinary cytokine levels

The study will have four arms including obese survivors of childhood brain tumors (OBT), lean survivors of childhood brain tumors (LBT), obese children with no history of tumors (ONB), and lean children with no history of tumors (LNB).

We will quantify cytokine concentrations in the serum and urine using the Bioplex ELISA kits (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 manufacturer's instructions. Cells will be sorted based on monocyte markers CD14 and CD16.

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

TLR pathway gene expression profiling looking at 84 genes involved in TLR expression and signaling pathway will be done using Human Toll-Like Receptor Signaling Pathway PCR Array (SABiosciences) as per manufacturer's instructions.

Determination of Methylation patterns of monocyte TLR pathway and cytokine genes

We will isolate genomic DNA from monocytes using DNAeasy Mini kit (Qiagen) as per manufacturer's instructions. The DNA will then be processed for qRT-PCR reaction using SYBR Green reaction master mix to measure methylation patterns for 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, Orthopedics and Cardiology services. The Neurooncology

program at our institution cares for 270 survivors of childhood brain tumors and the program reviews patients annually or more frequently depending of the tumor type and time from completion of therapy, with clinics serving 8 patients per week. The Orthopedics clinic serves 70-80 patients per week and the Cardiology service performs assessments of 24 patients per week. Assuming 50% recruitment rate based on fulfillment of inclusion criteria and interest in participation, our goal is to recruit 150 lean and obese brain tumor survivors and 150 lean and obese children with no history of tumors, at a rate of three patients per week over a two-year period.

Based on these figures, we estimate to have 99.7% power to reject the null hypothesis with alpha set at 0.05, and the difference in population means is 12 pg/ml, and a difference within a group of 21.9 pg/ml. The latter figure is set based on a previous study with one cytokine to determine the presence of inflammation in a sample of lean and obese children (unpublished). Importantly, to obtain 80% power, we will need 29 participants in each of the groups, and these calculations are done using Power and Sample Size Calculations software version 3.0.43 [96]. Our overall recruitment target is consistent with reported enrollment rates of children with cancer in clinical trials, with rates ranging from 70% participation rate in the 0-14 year old group [97] and dropping to 24% in 15-19 year old category, and other earlier studies demonstrating even lower rates of recruitment in the latter age group [98-100].

There is conflicting evidence regarding the participation of ethnic minorities in pediatric cancer studies, with some showing appropriate representation while

others documenting underrepresentation [101-103]. 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 summarized using descriptive summary measures expressed as mean (standard deviation) or median (minimum-maximum) for continuous variables and number (percent) for categorical variables. In addition, we will test for differences in sociodemographic and baseline clinical characteristics between groups using chi-square tests for categorical variables and one-way analysis of variance (ANOVA) 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 95% confidence interval and associated p-values. Statistical significance will be set at alpha = 0.05 adjusted using Bonferroni approach for multiple analyses. We will examine the residuals to assess model assumptions. All analyses will be performed using SAS 9.2 (Cary, NC) or SPSS (Chicago, IL) statistical software.

Discussion

In this study, we will investigate the mechanisms of inflammation in obese survivors of childhood brain tumors and compare them to lean survivors and lean and obese children with no history of tumors.

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 pediatric age group and express inflammatory markers, then interventions may have to be more aggressive including life style intervention, nutraceutical and pharmacological treatments to address these mechanisms.

Understanding 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 randomized controlled trial using nutraceutical of pharmacological therapy with or without life style intervention to identify the most effective intervention(s) in ameliorating inflammation, and if this occur via modulation of 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 the lean survivors or lean and obese children in the non-tumor 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 tumors, and 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 co-morbid associations.

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Authors' contribution: MCS conceived the study idea and generated the hypotheses. MCS, LT, RFD, SB, and KS finalized the study design, and MCS and LT completed the statistical analysis plans. MCS, RFD, SB, and KS contributed to the definition of study cohorts, inclusion and exclusion criteria, recruitment plan, and study logistics including space and resource allocation. MCS wrote the manuscript draft and all authors reviewed the current version.

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Table 1: CanDECIDE study primary objectives and statistical analysis plan.

Table 2: CanDECIDE study secondary objectives and statistical analysis plan.

Figure 1: Flow chart of the 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 enrollment. The participants will be stratified into the four arms of the study including obese childhood survivors of brain tumors (OBT), lean childhood survivors of brain tumors (LBT), obese children with no history of tumors (ONB), and lean children with no history of tumors (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.

Table 1: CanDECIDE study primary objectives and statistical analysis plans

Objectives	Outcome	Independent variables	Hypotheses	Statistical analysis
Primary				
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 tumors and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors	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 versus controls	1. Age 2. Sex 3. Ethnicity 4. Puberty 5. Tumor type 6. Tumor location 7. Tumor treatment 8. Hormonal deficiencies 9. BMI 10. Lipid levels 11. HOMA-IR	Obese survivors of childhood brain tumors have higher inflammatory status and altered gene methylation patterns compared to lean survivors and non-cancer controls that predisposes them to Endometabolic risks	Regression

Comment [MS20]: AS REQUESTED

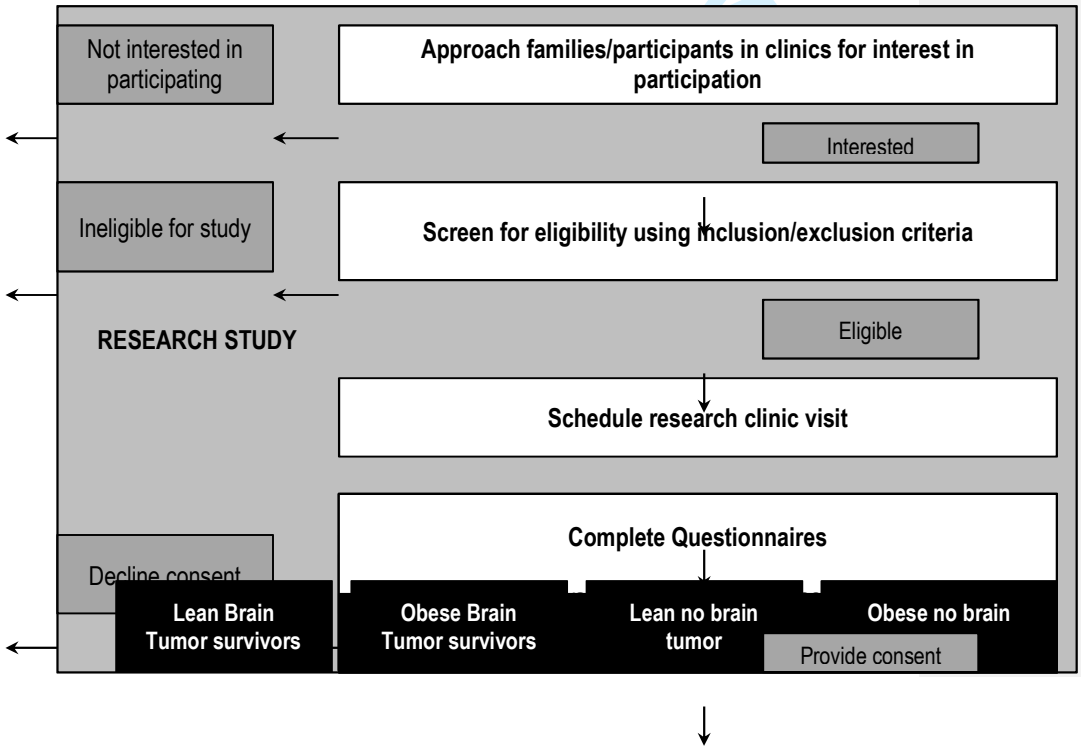
Table 2: CanDECIDE study secondary objectives and statistical analysis plans

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 tumors and comparing them to non-obese tumor survivors and lean and obese subjects with no history of tumors.	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 to non-obese tumor survivors and lean and obese subjects with no history of tumors.	1. Age 2. Sex 3. Ethnicity 4. Puberty 5. Physical activity 6. Tumor type 7. Tumor location 8. Tumor treatment 9. Hormonal deficiencies 10. BMI 11. Lipid levels 12. HOMA-IR	Obese survivors of childhood brain tumors have higher inflammatory status and altered gene methylation patterns, and this is mediated via individual and lifestyle factors	Descriptive analysis Regression

Comment [MS21]: AS REQUESTED

Figure 1



Article summary:

Article focus:

- The goal of CanDECIDE study is to determine if obese survivors of childhood brain tumors have more inflammation than lean survivors, and lean and obese children with no history of tumors.

- It will also evaluate the potential mechanisms involved in the occurrence of inflammation and immune system activation in these groups.

Key messages:

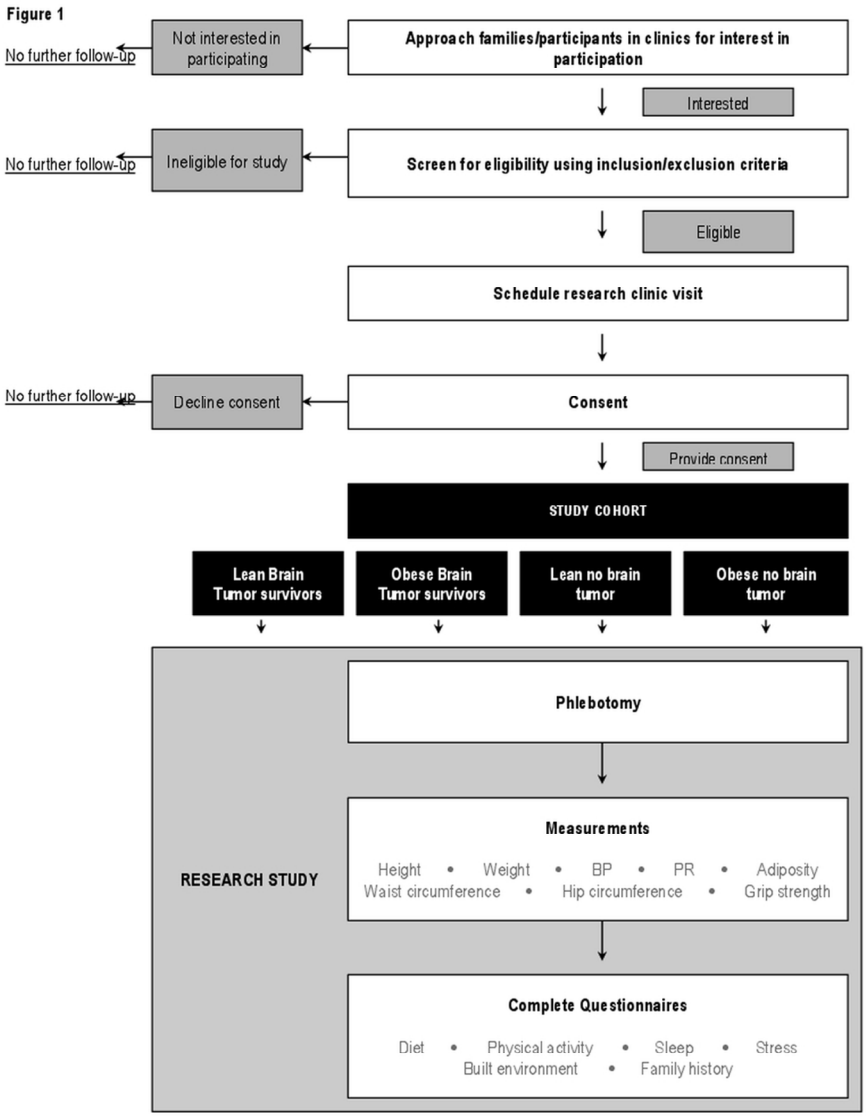
-This study will determine the inflammation and immune system activation status of obese survivors of childhood brain tumors and children with no history of cancer.

-This may allow the determination of preventative and therapeutic strategies to mitigate the risk of obesity and its comorbidities in this population.

Strengths & limitations of the study:

-The strength of this study is that it will systematically study the inflammatory response in childhood obesity in pediatric brain tumor 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.



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