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GENOME INSTABILITY IN CHILDHOOD OBESITY

A CONCEPTUAL FRAMEWORK FOR AN ASSESSMENT, INTERVENTION AND MONITORING PROGRAMME OF INFLAMMATION AND DNA DAMAGE IN PAEDIATRIC OBESITY

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ABSTRACT

Purpose: Recent studies have raised concerns that obese children may present an increased pre-disposition towards age–related disorders such as cancer. Initial evidence would suggest that the increased risk of developing cancer later in life might be linked to the reported presence of chronic low–grade inflammation in childhood obesity and the sustained accumulation of Reactive Oxygen Species (ROS) capable of inflicting DNA damage, a well–known promoter and driver of carcinogenesis. This conceptual framework outlines the clinical and laboratory investigations required for the combined assessment and monitoring of systemic inflammation, micro–nutritional deficiencies and acquired genome damage in childhood obesity.

Approach: A case–control study is proposed in a cohort of obese and healthy weight 11–15 year olds. This study combines assessments of blood, together with minimally invasive investigations of saliva and urine samples to obtain an overview of inflammation and DNA damage status in obesity. Furthermore, we present a nutrition sensitive intervention programme to investigate the reversibility of these pathological states.

Findings: It is hypothesised that chronic inflammation in childhood obesity can establish a harmful microenvironment that inflicts damage to DNA. It is postulated that a nutrition sensitive intervention may reverse implications of inflammation and DNA damage in obese children.

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Value: An evaluation of the role of genomic instability and cumulative DNA damage early in life as a possible causative link between childhood obesity and the increased risk of developing cancer in adulthood. The conceptual framework proposes a personalised approach to disease diagnosis and monitoring of 'genome health' that can inform the prioritisation and severity of intervention measures in the clinical setting.

Keywords: Genome instability; Childhood Obesity; Nutrition sensitive intervention; DNA damage; Cancer; Chronic inflammation; Reactive Oxygen Species; Conceptual Framework

INTRODUCTION

Obesity is one of the most concerning medical and social burdens, believed to be causing a global economic impact of almost $2.0 trillion (McKinsey Global Institute, 2014). According to the World Health Organisation (WHO, 2014), obesity in adults has doubled across the globe over the last 25 years. However, the rates of obesity in children are far more concerning because obese children are more likely to become obese adults, perpetuating the obesity epidemic. Currently, almost a third of the children in the UK are obese (Health and Social Care Intervention Centre, 2015) and, despite intervention strategies, there are expected to be 60 million obese children across the globe by 2020 (De Onis et al., 2010). In principle, obesity is caused by an increase in caloric intake accompanied by a reduced expenditure of energy. Urbanisation, the increased use of television and technology and reduced participation in outdoor sports are further factors that have caused the childhood obesity epidemic (Malik et al., 2013). Childhood obesity is not only a problem for the UK and USA; it is estimated that 35 million obese children reside in developing countries (De Onis et al., 2010; Poobalan and Aucott, 2016).

The causes of obesity revolve around the influence of society on behaviour, certain co-existing medical conditions and genetics (Akabas et al., 2012). Society and the environment play a key role in the education of parents and children about living healthy lifestyles. Ethnic background and culture can impact behaviour as families feel obliged to follow certain traditions that may contradict with the 'modern healthy lifestyle' (Caprio et al., 2008). In addition to nutritional and behavioural causes, there are certain medical conditions that exhibit obesity in childhood. These are mainly endocrine disorders such as Cushing's syndrome, hypothyroidism, growth hormone deficiency and pseudohypoparathyroidism as reviewed by Han et al., (2010). Linkage studies have shown gene variants of the Fat mass and Obesity associated protein (FTO) (Dina et al., 2007), Melanocortin Receptor 4 (MC4R) (Larsen et al., 2005), and leptin receptor (Clément et al., 1998) relating to the development of obesity. More recently, a variant in the CD36 fatty acid transporter gene was also associated with early onset obesity (Solakivi et al., 2015). The emerging aetiological scenario is therefore much more complex than simply sloth and gluttony.

The persisting detrimental health impact of childhood obesity prevails as the focus of attention. An extensive review by Park et al., (2012) indicated that childhood obesity may predispose to a range of morbidities; these include type 2 diabetes, hypertension, chronic heart disease, asthma and cancer. In particular, an overweight/obese BMI ($\geq$30Kg/m^2) between ages 2 and 19 was reported to potentially increase the risk of acquiring colorectal, kidney, cervical and ovarian cancer in adulthood by up to 40% (Park et al., 2012). Further studies have indicated that childhood obesity may have an aetiological role in pancreatic (Nogueira et al., 2014) and oesophageal (Cooke et al., 2006) cancer in adulthood. Contrastingly, no association between childhood obesity and breast cancer later in life has been found so far (Jeffreys et al., 2004; Le Marchand et al., 1988; De Stavola et al., 2004). Indeed, it has also been suggested that childhood obesity can provide protection against premenopausal breast cancer (Chu et al., 1991). Given the expected rise in childhood obesity (De Onis et al., 2010), obtaining conclusive evidence on the long term consequences should be considered a priority.

CHRONIC INFLAMMATION IN CHILDHOOD OBESITY

The association of obesity with inflammation driven co-morbidities, including cancer in

adulthood, implies that childhood obesity itself may be an inflammatory disease (Schipper et al., 2012). Significant findings of plasma C-Reactive Protein (CRP) – an acute phase protein commonly used as a clinical risk marker of cardiovascular disease and activator of the complement mediated inflammatory response correlates with increasing visceral fat in obese children (Park et al., 2005). CRP has been found to be up to 270% higher in plasma of obese children when compared to their lean counterparts (Cook et al., 2000). Furthermore, pro-inflammatory cytokines including, TNF-a and MCP-1, may be substantially raised (34% and 22% respectively) in obese children relative to those who are lean (Breslin et al., 2012). This is in addition to ICAM-1, an adhesion molecule involved in the recruitment of inflammation promoting cells of the immune system (Tam et al., 2010). The recruitment of such cells and the increasing hypertrophy of adipocytes in childhood obesity has also been associated with an up to two–fold increase in the secretion of inflammation promoting molecules termed 'adipokines' directly from WAT, including chemerin and leptin (Schipper et al., 2012). Furthermore, a significant reduction in anti–inflammatory adipokines, such as Annexin-A1 (Kosicka et al., 2013) and adiponectin, has also been reported in severe obesity (Arnaiz et al., 2010). The above indications of a chronic low–grade inflammatory response and abnormal metabolic profile in obese children are of great concern. This is because chronic inflammation is a well-known etiological factor for DNA damage and, as a result, carcinogenesis (Hanahan and Weinberg, 2016).

INTERVENTIONS IN CHILDHOOD OBESITY

Parental education on health and well-being has been reviewed as a fundamental modern intervention strategy that can encourage positive behavioural changes in obese children (Jalali et al., 2016). Such family-based interventions can include counselling, dietary modifications and increments in the level of physical activity. Indeed, these interventions implemented alongside motivational interviews, stage-based goal-setting and parental training have shown evidence for establishing weight maintenance in children (Akabas et al., 2012). In the UK, organisations such as Mind, Exercise, Nutrition, Do it (MEND), MoreLife and TrimTots deliver healthy lifestyle programmes within communities, not only to treat but also to prevent childhood obesity. The 9-week MEND programme was evaluated in a Randomised Control Trial that determined its significant impact on waist circumference and Body Mass Index (BMI) reduction (Sacher et al., 2010). These findings suggest that a successful intervention programme against obesity requires a combined focus on nutrition and behavioural modification as recommended in the Childhood Obesity Prevention (CHOP) framework (Tewfik, 2008).

However, achieving and maintaining weight-loss in extreme obesity can be more challenging. The Food and Drug Administration (FDA) approved drug Orlistat can be used to treat adolescent obesity; however, side-effects such as malabsorption of essential fat soluble vitamins from the gut is a cause for concern (Kanekar and Sharma, 2010). When conventional methods of treatment fail, most often due to non-compliance (Inge et al., 2007), surgical interventions for extreme childhood obesity may be required.

More recently, gastric bypass for adolescents with severe obesity (BMI above 40Kg/m2) or with serious co-morbidities, has been acknowledged as an effective method for weight-loss to improve the quality of life (Widhalm and Helk, 2015). It has been suggested that bariatric surgery as a weight loss intervention can be more beneficial for reducing obesity related co-morbidities when implemented in adolescence rather than adulthood (Inge et al., 2007). However, bariatric surgery in children requires proceeding with caution: as it is an invasive intervention there are a number of moral issues that require addressing before the procedure can be employed (Hofmann, 2013).

Since bariatric surgery prior to a progression of severe co-morbidities has positive implications on markers of chronic diseases such as Cardio Vascular Disease (CVD), Obstructive Sleep Apnoea Syndrome (OSA), Diabetes and Non-Alcoholic Fatty Liver Disease (NAFLD) (Inge et al., 2007), there is the likelihood of an impact on systemic inflammation and DNA damage. Preliminary research findings have identified that calorie-restriction following surgery may increase

telomere length and reduce the number of abasic sites in the Rectal Mucosa (RM) (O'Callaghan et al., 2009). Therefore, further investigations into the long-term benefits of bariatric surgery are very necessary, especially because surgery may be the last resort for adolescents struggling to achieve weight loss conservatively. If bariatric surgery can improve markers of inflammation and maintain genome health in severely obese adolescents, then this method of weight-loss may possess novel implications for cancer prevention.

RESEARCH RATIONALE AND HYPOTHESIS

It is evident that childhood obesity remains one of the most serious global public health challenges. Therefore, establishing its lifelong consequences on health could not be any more urgent. It is concerning that recent studies have suggested that obese children not only have a reduced life expectancy, but a pre-disposition to age related disorders, including cancer (Park et al., 2012). Evidence of a chronic and systemic meta-inflammatory state in obese children has been identified (Schipper et al., 2012). However, the risks of such inflammation contributing to further morbidities in obese children are not clear. An hypothesis has been formulated that chronic inflammation in childhood obesity can establish a harmful micro-environment that inflicts damage to DNA. It is also postulated that obesity-acquired genomic instability may be detrimental, increasing the risk of cancer in childhood, but also pre-disposing severely obese children to developing cancer later in life.

Therefore, it is relevant to investigate acquired DNA damage in early-onset obesity. This investigation can aid the identification of pre-cancerous genetic changes and consequently allow the monitoring of pre-pathological conditions in the morbidly obese child. Ultimately, this investigation will help to establish the relationship between childhood obesity and morbidity later in life, and elucidate the reversibility of pre-cancerous developments by applying a nutrition-sensitive intervention programme.

AIMS OF THE STUDY

1. To evaluate the role of genomic instability as a possible causative link between childhood obesity and the increased likelihood of cancer in adulthood;
2. To establish the applicability of personalised, minimally invasive early detection of acquired genome damage as a pre-cancerous biomarker in obesity and otherpre-pathological conditions;
3. To evaluate the effectiveness of a nutritional and behaviour modification model in decreasing obesity, inflammation and DNA damage among teenage children.

SPECIFIC OBJECTIVES OF THE STUDY

1. To validate a non-invasive, follow-up protocol for early detection and monitoring of genomic instability in childhood;
2. To conduct a case-control study (severely obese vs healthy weight) combining analysis of systemic inflammation and micro-nutritional deficiencies with the assessment of acquired genome damage;
3. To investigate the reversibility of acquired genome damage in childhood obesity by implementing a behaviour and nutrition sensitive intervention programme.

SUBJECTS, MATERIALS AND METHODS

Study Area and Settings

A target population of participants affected by severe obesity is to be recruited from weight management clinics. Control participants should be healthy weight (BMI between 5^{th} and 85^{th} percentile) and matched in age and gender to the target population. Possible settings to recruit a random sample of control participants include, but are not limited to, schools, faith centres and activity/youth clubs.

Study Design and Subjects

It is recommended that this study is conducted in the following consecutive phases;

1. Establish collaborations with local obesity clinics;
2. Develop participant and parent consent forms, information booklets, data capture forms and educational materials;
3. Establish laboratory markers for DNA damage and inflammation assessment, and optimise laboratory techniques for the relevant assays;
4. Submission of ethical approval to the relevant committees;
5. A pilot study to establish pre-assessment, intervention and post-assessment protocols;
6. Monitoring and amendment of protocols in preparation for scale-up phase;
7. Scale-up the pre-assessment, intervention and assessment phases;
8. Evaluation of the programme;
9. Conduct a sustainability assessment and dissemination plan.

STUDY PHASES (FIGURE 1)

Planning (Preparatory) Phase

1. Finalise collaborative arrangements;
2. Obtain ethical approval from relevant bodies;
3. Preparation of participation information booklets and consent forms;
4. Preparation of data capture forms;
5. Preparation of health and behaviour educational materials;
6. Optimisation of laboratory techniques and assays.

Pilot Phase

1. Pre-intervention assessments:
   - Anthropometric analysis including body fat percentage (via TANITA), waist:hip ratio, height and weight;
   - Nutritional intake assessment;
   - Inflammation assessment – Saliva for (CRP) assay;
   - DNA damage assessment – obtain cheek swab for Buccal Micronucleus Cytome Assay (BMCA), DNA extraction from saliva and quantitative Polymerase Chain Reaction (qPCR) assay for telomere length, urinary 8-OH-2-Deoxy Guanosine (8-OHdG) test;
   - Micro-Nutritional Deficiency test – Plasma and Saliva vitamin D.
2. CHOP Intervention (Tewfik, 2008):
   - Health education for parents and child – nutrition and behavioural models;
   - Establish action on nutrition – reduction in fat, sugar and salt and increased intake of fresh fruit and vegetables;
   - Establish action on behaviour – limit screen time to $<$2 hours per day and increase levels of moderate to vigorous physical activity to at least 60 minutes per day.
3. Post-intervention assessments:
   - Repeat all anthropometric and clinical measurements from pre-assessment stage.

Scale-Up Phase

1. Conduct pre-assessment, intervention and post-assessment programme in total sample size under appropriate considerations and recommendations obtained from pilot phase.

Evaluation, Sustainability and Dissemination Phase

1. A formative evaluation of the programme should take place to consider whether the spe cified objectives have been met and how the programme can be further developed and sustained;
2. A dissemination plan should be established at this stage to further the integration of the programme.

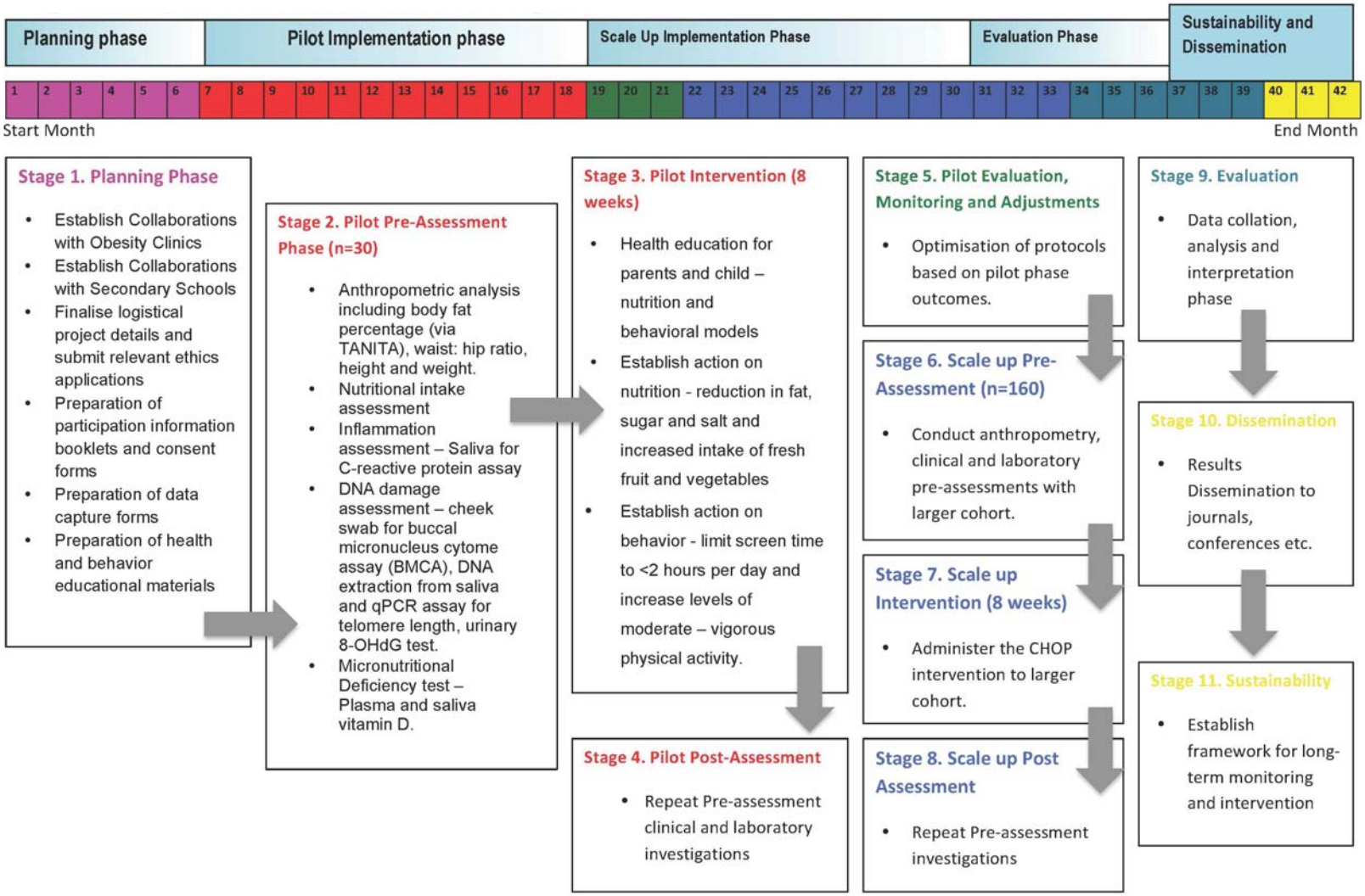

Figure 1 Conceptual Framework for GICO Intervention Programme

SAMPLE SIZE AND STATISTICAL ANALYSIS

Sample size should be calculated by first determining the standard deviation values of the primary end points in the control population and then in a population of patients with cancer (age-matched to the control population). The standard deviation values should be *log transformed* before a comparison of the means *a priori* analysis takes place in a statistical software such as GPower v3.1.3. For three primary end points, p should equal to 0.017 to achieve an overall total error rate of 5%. Statistical analysis should include two tailed t tests, with the Bonferroni correction applied.

DATA COLLECTION METHODS, INSTRUMENTS USED, MEASUREMENTS

Data Collection Instruments

Questionnaire

Following the consent process, participants can be screened using a medical questionnaire to record:

- Age;
- Gender;
- Ethnicity;
- History of exposure to X-rays (warrants likelihood of DNA damage to buccal epithelial cells);
- Oral condition and history of dental treatment (warrants possible increase in salivary CRP);
- Food and drink intake;
- Physical activity levels;
- Nutritional assessment;
- Medical conditions;
- Drug intake (including micro-nutrient and herbal supplementations);
- Travel history (to determine sun exposure for vitamin D status);
- Fitzpatrick Skin typing test (Fitzpatrick, 1988).

Anthropometrical Measurements

A compre-hensive anthropometric assessment will consist of recording height, weight, body fat percentage and waist and hip circumference.

Health Education Materials

Health education materials for school children and similar materials for their parents can be prepared to inform them about the effects of diet, physical activity and lifestyle in relation to health.

Sample Collection Manual

A workflow with participants should be prepared for clinicians and trained researchers, detailing the occurrence of anthropometrical measures, sample collection and storage conditions.

Sample Collection Instruments

Buccal Epithelial Swab – A small brush, spatula or tooth pick can be used to collect a sample of epithelial cells from the inner cheek, although the first of these has been described to be the most effective (Thomas et al., 2009). Samples must be stored at 4°c in Saccomanno's fixative.

Saliva (Passive Drool) – Required for the isolation and purification of salivary DNA. A large number of commercially available kits can be employed for this purpose, with preservative vials allowing the storage of samples at room temperature.

Saliva (Swab) – A cotton swab to be chewed by the participant to yield enough saliva for vitamin D analysis and CRP.

Urine – Ideally a first morning, mid-stream urine sample should be collected for the analysis of 8-OHdG.

Plasma samples – A trained phlebotomist is to collect a standard blood sample for analysis of hs-CRP.

LABORATORY ASSESSMENTS

Inflammation

CRP has been selected as the marker of choice to obtain an assessment of systemic chronic inflammation. CRP is an acute phase protein that is produced by the liver in the early stages of an inflammatory response. Chronic inflammation coincides with persisting CRP levels in plasma, allowing it to be clinically utilised as a non-specific biomarker of inflammatory conditions, including cardiovascular disease (Shrivastava et al., 2015) and cancer (Trichopoulos et al., 2006). Moreover, CRP is measurable in saliva and may be secreted

in higher levels in overweight children (Naidoo et al., 2012). These factors increase the suitability of CRP as a non-invasive biomarker for monitoring inflammation, although other possible markers of inflammation in saliva may include cortisol. Cortisol is an anti-inflammatory hormone that is increasingly abundant in saliva and poses good correlation with serum levels. However, Adam and Kumari (2016) have reviewed the use of cortisol in large epidemiological studies and suggest the major fluctuations in this hormone throughout the day can cause logistical sampling issues.

A sandwich Enzyme-Linked Immunosorbent Assay (ELISA), using a commercially available high sensitivity ELISA Kit, can be used to quantitatively measure salivary CRP. The assay must be carried out as per manufacturer's instructions.

Genomic Instability

A combination of the following well-established laboratory tests can be applied to assess DNA damage:

BUCCAL MICRONUCLEUS CYTOME ASSAY (BMCA)

A micro-nucleus is a marker of chromosomal instability as it carries chromosomal fragments that have failed to become incorporated into the main nucleus during mitosis. Micronuclei frequency has been associated with many types of cancers including those of the blood (Bonassi et al., 2006) and gastrointestinal tract (Maffei et al., 2014). A micro-nucleus test can be set up according to the protocol of Thomas et al., (2009), which involves fixation of buccal epithelial cells harvested from the cheek swab onto a microscopic slide with Feulgen and Light Green stains. The cells can be examined at a x1000 magnification using a fluorescence microscope with a far-red filter, classified according to their morphological features and scored according to criteria established by Thomas et al., (2009).

Urinary 8-OHdG

8-OH-2-deoxy Guanosine (8-OHdG) is a mutagenic DNA lesion that is generated following oxidative damage to guanosine (Wu et al., 2004). High levels (>10.0ng/mg) of urinary 8-OHdG have been documented in obese adolescents (El Wakkad et al., 2011) and in children with leukaemia (>8.00ng/mg) (Yang et al., 2009). A commercially available ELISA kit can aid the detection of 8-OHdG in urine samples, although 8-OHdG can also be measured in plasma.

qPCR Telomere Length

Telomeres are repetitive segments of non-coding DNA located at the ends of chromosomes that serve a protective function (De Lange, 1995). The shortening of telomeres can be described as a marker of cellular ageing (Allsopp et al., 1992) and consequently age-associated pathologies including cancer (Broberg et al., 2005). Telomere shortening is a phenomenon that has been previously discovered in Peripheral Blood Lymphocytes (PBLs) of a large cohort of obese children (Buxton et al., 2011). This indicates that chromosomal instability is a possible pre-pathological condition in obese children that requires a deeper investigation. Recent population studies have successfully applied DNA obtained from saliva for telomere length quantification (Theall et al., 2013). Saliva can be collected via the passive drool method to isolate DNA. Following DNA purification, relative telomere length can be measured using a quantitative Polymerase Chain Reaction (qPCR). Telomere lengths will be determined upon the repeat copy number to single gene copy number (T/S) ratio and compared to the control group as described by Cawthon (2002).

Micro Nutritional Deficiency

The potential role of vitamin D in DNA repair and carcinogenesis (Nemazannikova et al., 2014) calls for the assessment of this micro-nutrient in this study. Moreover, an increased BMI has been described as a causative factor for vitamin D deficiency (Vimaleswaran et al., 2013). 25-hydroxy Vitamin D levels should be assayed in plasma in order to obtain a more accurate representation of vitamin D status. For the purpose of long-term monitoring via a minimally invasive approach, vitamin D detection in saliva can be simultaneously explored using an ELISA-based technique.

QUALITY CONTROL MEASURES AND GOOD PRACTICE FOLLOWED DURING STUDY IMPLEMENTATION

All procedures associated with the processing and analysis of saliva and urine samples should be conducted in accordance with recognised standard operation procedures and under complete aseptic conditions and health and safety requirements.

MODEL OF INTERVENTION

The model of intervention in this study follows that of the CHOP framework (Tewfik, 2008), which advocates these four main behavioural modifications:

1. Reduction in screen viewing to less than two hours per day;
2. Increasing the levels of moderate and vigorous physical activity;
3. Decreasing consumption of fat, sugar and salt;
4. Increasing consumption of fruit and vegetables to five a day or more.

ETHICAL CONSIDERATIONS

Due to the participation of children, careful consideration of ethical issues should take place and approval should be sought from the relevant committees. To minimise the ethical considerations, it should be made clear that no whole genome sequencing is required and, where possible, blood samples from patients should only be collected on 'opportunistic' occasions. Thereby, blood samples should be retrieved within the child's clinic and only as part of a routine blood test. Furthermore, relevant participant and parent information sheets must be provided for all subjects and written parental/guardian consent should be sought. Ethical approval may also be required from the research hosting educational institution.

CONCLUSION

Implementation of this conceptual framework over a period of three years can enable a thorough investigation of inflammation and status of DNA stability across a cohort of obese adolescents. This document also provides the framework for a nutrition sensitive intervention that can be applied to assess and monitor the effectiveness of weight loss with respect to systemic inflammation and DNA stability. We recommend this research study to be implemented as a pilot investigation first, in order to assess and successfully satisfy the ethical considerations and ensure laboratory conditions and tests be optimised prior to scale-up.

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BIOGRAPHICAL NOTES

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