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Effect of Blueberry Supplementation in Overweight and Obese Pregnant Women

Thesis of

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ABSTRACT AND GOAL OF THE THESIS

Childhood obesity is a serious problem growing worldwide that needs to be early considered and treated. Maternal overweight and obesity lead to foetal complications and to an increased risk of child obesity. Blueberry bio-active compounds have antioxidant properties and improve insulin sensitivity in obese individuals. We proposed a daily integration of a freeze-dried blueberry extract in the last trimester of a population of overweight and obese pregnant women. Mother cytokine milieu, birth weight and at age one, were compared with a matched control group.

Results: birth weight was significantly lower in those whose mothers assumed the blueberry extract (male: 3636 gr ± 56,7 in control group and 3302 gr ± 60,9 in the blueberry, P=.001; female: 3446 gr ± 53 in control group and 3094 gr ± 65,5 in the blueberry, P<.0001) and this result remained comparable at age one (male control group: 9597 gr ± 134,4; blueberry: 8656 gr ± 271,9; P=.0021; female control group: 8812 ± 167,5; blueberry: 8083 ± 266,3; P=.0211); pro-inflammatory cytokines (TNF-α and leptin) were significantly reduced in the blueberry group (control 29,89 pg/mL ± 0,283 vs blueberry 8,124 ng/mL ± 0,1085 and 17,28 ± 0,4742 vs 6,977 ± 0,1187, respectively); anti-inflammatory cytokines (TGF-β and IL-4) were significantly increased in the blueberry group (control 4,196 pg/ml ± 0,9752 vs blueberry 14,87 pg/ml ± 0,8844 and 14,87 pg/ml ± 0,8844 vs 55,22 ± 1,47, respectively), CRP levels were significantly lower in the blueberry group (control 5,917 mg/L ± 0,698 vs blueberry 3,741 mg/L ± 0,6279).

Conclusion: a daily dietary supplementation with bioactive from whole blueberries in overweight and/or obese pregnant women is an effective intervention to reduce the risk of foetal macrosomia and early childhood obesity.

ABBREVIATIONS

BMI	Body Mass Index
HFD	High Fat Diet
CRP	C-Reactive Protein
S3PIO	Swiss Project for Precocious Prevention of Infantile Obesity
TNF-α	Tumour Necrosis Factor Alfa
IFN-γ	Interferon Gamma
IL-4	Interleukin 4
TGF-β	Tumour Growth Factor beta
ELIZA	Enzyme-linked Immunosorbent Assay
GI	Gastrointestinal
PYY	Peptide YY
IR	Insulin Receptor
LR	Leptin Receptor
NPY	Neuropeptide
AGRP	Agouti-related Protein
POMC	Pro-opiomelanocortin
CART	Cocain-amphetamine Related Transcript
PC-1	Prohormone Convertase One
CPE	Carboxypeptidase E
MSH	Melanocyte Stimulating Hormone
TRH	Thyrotropin Stimulating Hormone
MCH	Melanin Concentrating Hormone
GABA	Gaba-Amino Butyric Acid
BDNF	Brain Derived Neurotropic Factor
NCD	Non Communicable Disease

1. Introduction

a. Epidemiology

Childhood overweight and obesity are global burden diseases. The prevalence of this disorders increased from 4,2% in 1963-65 to 15,3% in 1990-2000 and have plateaued during the first decade of the 21st century[1].

The Italian registry of overweight and obesity showed that in year 2016 21,3% of children were overweight and 9,3% were obese with higher incidence in the southern part of the country (see **Figure 1 and 2**)[2].

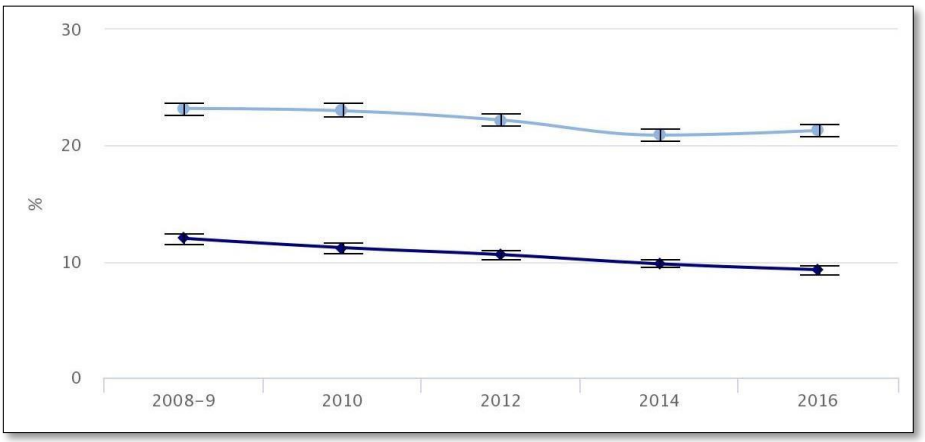


Figure 1: *Italian childhood overweight and obesity trend in the last 8 years. Note that overweight interests ca. 21,3% of the paediatric population and obesity ca. 9,3%. Fortunately the trend is favourable and the prevalence is slightly decreasing.*

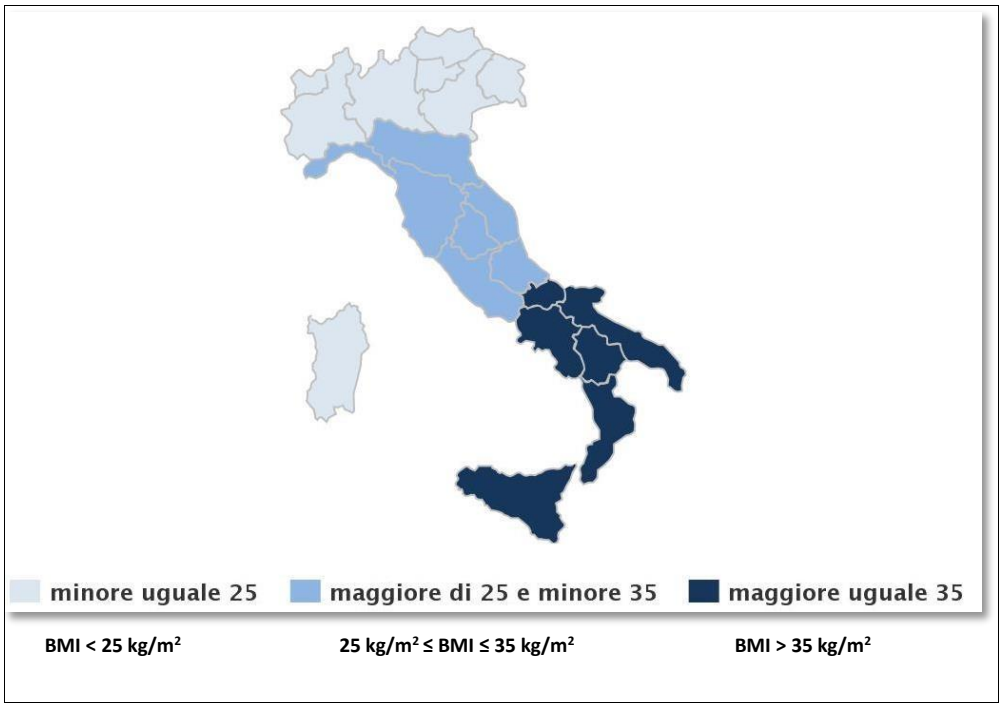


Figure 2: *Higher BMIs are present in the central and southern part of the country.*

The Swiss registry for overweight in 2017 registered a prevalence of 20% of overweight children and 7% of obese children[3].

b. Definition of childhood obesity

There are internationally agreed threshold of body-mass index (BMI) for defining under-weight, normal-weight, overweight and obesity in adults, but in children, the marked effects of age, gender, pubertal status and race/ethnicity on growth make classification difficult. Commonly used cut points for childhood overweight and obesity include: 110% or 120% of ideal weight for height; weight-forheight Z-scores of >1 and >2, and BMI at the 85th, 90th, 95th and 97th percentiles (based on international or country-specific reference populations, see **Table 1**).

Age	**0-2 years**	**2-5 years**	**5-18 years**
Marker	Weight for height	BMI	BMI
Reference	OMS 2006	OMS 2006	OMS 2007
> 85th Percentile	Overweight risk	Overweight risk	Overweight
> 97th Percentile	Overweight	Overweight	Obesity

> 99th Percentile	Obesity	Obesity	Severe obesity

Table 1: Diagnostic criteria for childhood overweight and obesity.

c. Determinants and risk factors for childhood obesity

Overweight is a complex condition which is influenced by a wide-range of genetic and non-genetic factors, with interactions between many of these. **Figure 3** shows a simplified model of the leptin signalling pathway, the key biological pathway that controls energy balance.

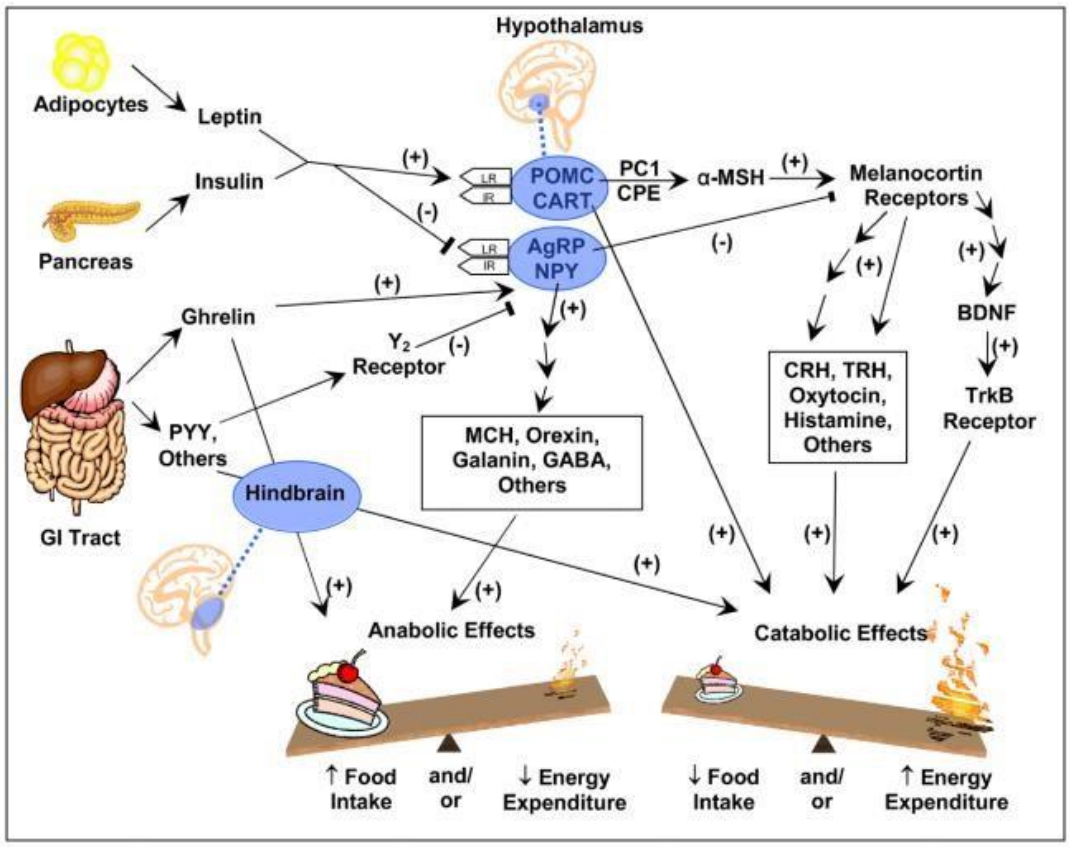


Figure 3: *Both insulin and leptin are secreted in proportion to body fatness and serve as adiposity signals, acting on the same neurons of the hypothalamic arcuate nucleus to regulate energy homeostasis. Ghrelin, which is secreted by the stomach and duodenum, serves as a hunger signal at the hypothalamus and brainstem, while other peptides secreted by the GI tract, including PYY, act as satiation signals. The ligands leptin, POMC, CART and BDNF, the receptors for leptin, melanocortins, and BDNF, and the enzyme PC1 have been found to have function-altering mutations associated with obesity in children. Mutations in the ligands and receptors for NPY, AGRP, CPE and MCH have been found to alter energy balance in rodents, but have not been as convincingly shown to be associated with human obesity. Lines with arrowheads indicate stimulatory action. Lines ending with a perpendicular end-block indicate inhibitory action.*

d. Complications associated with childhood obesity

Obesity can adversely affect nearly every organ system (see **Figure 4**) and often cause serious consequences, including hypertension, dyslipidaemia, insulin-resistance/diabetes, fatty liver disease and psychosocial complications[4].

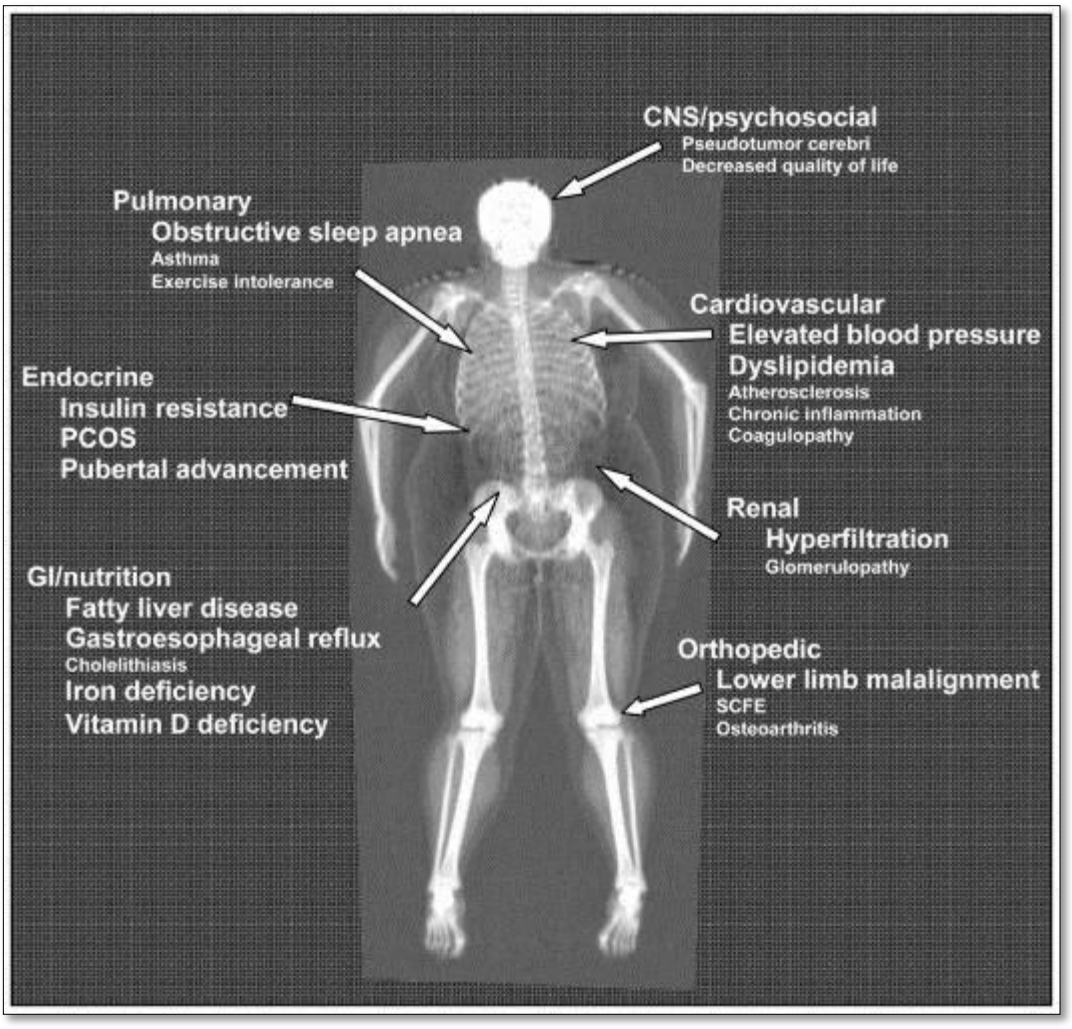


Figure 4: *Image obtained by dual energy X-ray absorptiometry from a teenage girl with BMI 38 kg/m2. Conditions that are of high prevalence and are well-established in their association with childhood obesity are shown in larger font size.*

Weight gain result in lipid accumulation and adipocyte stress, factors known to disrupt the balance of systemic cell signalling (adipokines and cytokines).

Macrophage accumulation in proportion to adipocyte size may increase the adipose tissue production of these proinflammatory and acute-phase molecules; since the cross talk between macrophages and adipocytes may negatively influence cellular insulin sensitivity[5] this consequently aggravates the obesity state[6,7] (see **Figure 5**).

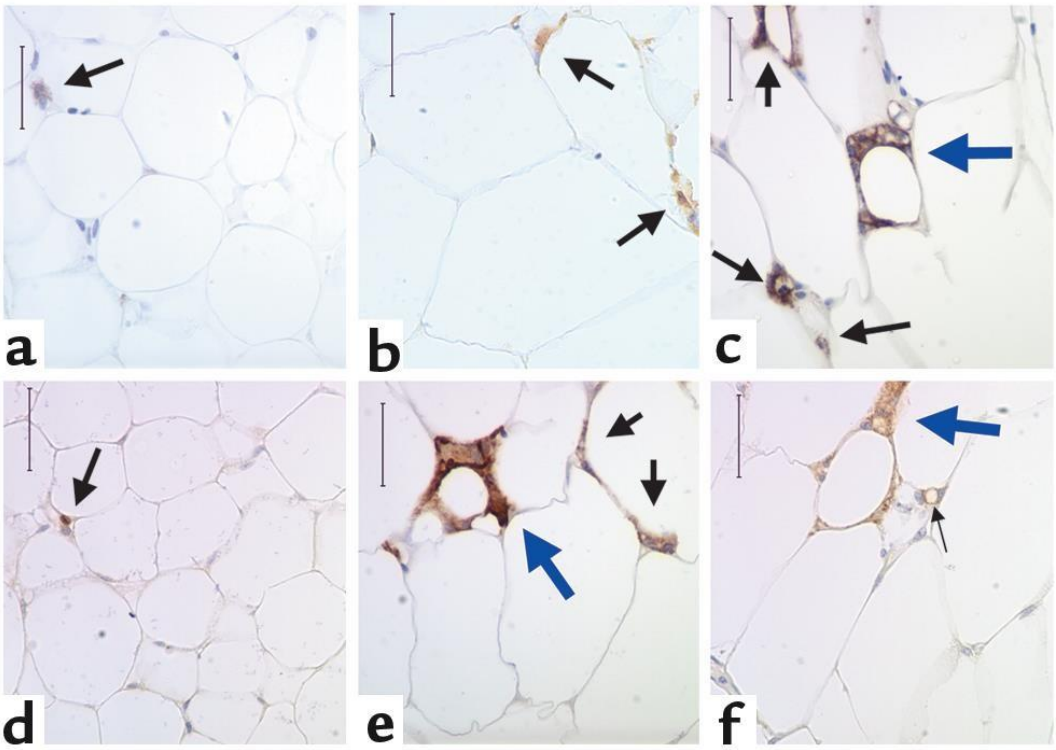


Figure 5. *Adipose tissue macrophages in mice with varying degrees of adiposity. Adipose tissue macrophage accumulation is directly proportional to measures of adiposity in mice and humans. Adipose tissue produces several proinflammatory, procoagulant, and acute-phase molecules in direct proportion to adiposity. Among these molecules, TNF-α, IL-6, PAI-1, NO, factor VII, and MCP-1 have been implicated in the development of adverse pathophysiological phenotypes associated with obesity.*

e. Treatment and prevention strategies

The current role-model of treatment of an obese children is multidisciplinary and focuses on a non-pharmacological; pharmacological and eventually surgical treatment[8].

The Italian Swiss reality offers two specific multidisciplinary program for the care of children with adiposity; one is called OTTIMIX® (see **Figure 6**) (dedicated to groups of children aged < 8 years) and the second is called ADOFIX® (see **Figure 7**), an individual program directed to older children.

Our multidisciplinary team is composed by a paediatrician (with a specific formation on childhood adiposity); a dietician; a psychologist; a physiotherapist; a ergo-therapist; an educator; a chef and an art-therapist. These programs integrate a larger Swiss program called "S3PIO" (Swiss Project for Precocious Prevention of Infantile Obesity).

With the large number of personnel and intensity of interventions, these programs usually have high operating expenses and are exacerbated by low recruitment rates or high rates of attrition[9]. Therefore, prevention, especially in the young, is universally viewed as the best approach for reversing the rising global prevalence of obesity[10].

***Figure 6**. Group program for overweight and obese children in the Italian Region of Switzerland; this program is dedicated to younger children (< 8 years) and it is active since 2012.*

Figure 7. *Individual program for overweight and obese children; this program is dedicated to older children (8-16 years old) and it is active since 2016.*

f. The first 1000 days

Particular opportunities for effective prevention of obesity and associated non-communicable diseases (*NCDs*) exist in pregnancy, in infancy and in early childhood; the first 1000 days, that describes the period from conception through age 2 years, is increasingly recognized as a critical period for development of childhood obesity and its adverse consequences[11].

Specifically, higher maternal pre-pregnancy BMI and excessive gestational weight gain have a consistent relationship with offspring overweight later in childhood and with adverse pregnancy outcomes[12].

The antenatal period, with opportunities for regular contact with health professionals, is considered an ideal time to intervene because mothers are motivated to make changes that could optimise their outcome and that of the baby[13].

g. The "Developmental origins of health and disease (DOHaD)" paradigm

The recent and rapid worldwide increase in NCDs, challenges the assumption that genetic factors are the primary contributors to such disease[14].

The "DOHaD" paradigm states that the environment during the preconception, gestation and lactation period shapes the developing individuals leading, in the case of a deleterious environment, to a predisposition to adult-onset diseases (see **Figure 8**).

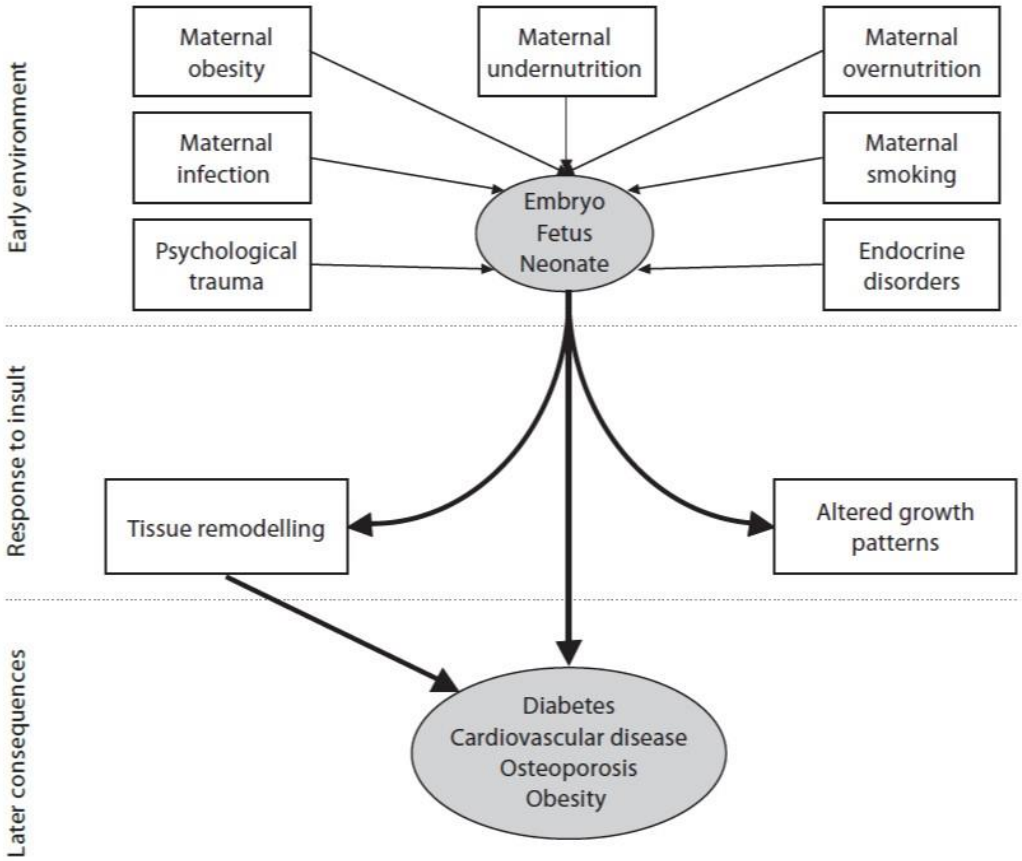


Figure 8: 1. The foetal origins of adult disease hypothesis. Adverse environmental cues from the mother are signalled to the developing foetus. The prevailing conditions may be sufficiently harsh to result in the loss of the pregnancy, or alternatively the foetus may mount adaptive responses to ensure immediate survival. One of these responses may be a slowing of growth that will ultimately result in lower weight at delivery. Other aspects of the adaptive response, which may be localized to specific organs and tissue types, will serve to modify physiology and metabolism, and hence tissue functions. The trade-off for overcoming the challenge in foetal life may be increased risk of disease in later life.

This theory was popularised by D.J. Barker in the early 1990s[15] and has recently become a famous topic (see **Figure 9**).

Figure 9. *"TIME" cover in 2010. The "DOHaD" paradigm cited by Barker received media consideration that dedicated a cover in the famous American news magazine.*

h. Epigenetics

More and more studies are converging to propose that epigenetics may be one of the key molecular mechanisms underlying the developmental programming of the phenotype.

Epigenetics refers to the field of science studying the heritable mechanism regulating gene expression without changing in the DNA sequence itself. There are two main physio-pathological mechanism that are illustrated and commented in **Figure 9**.

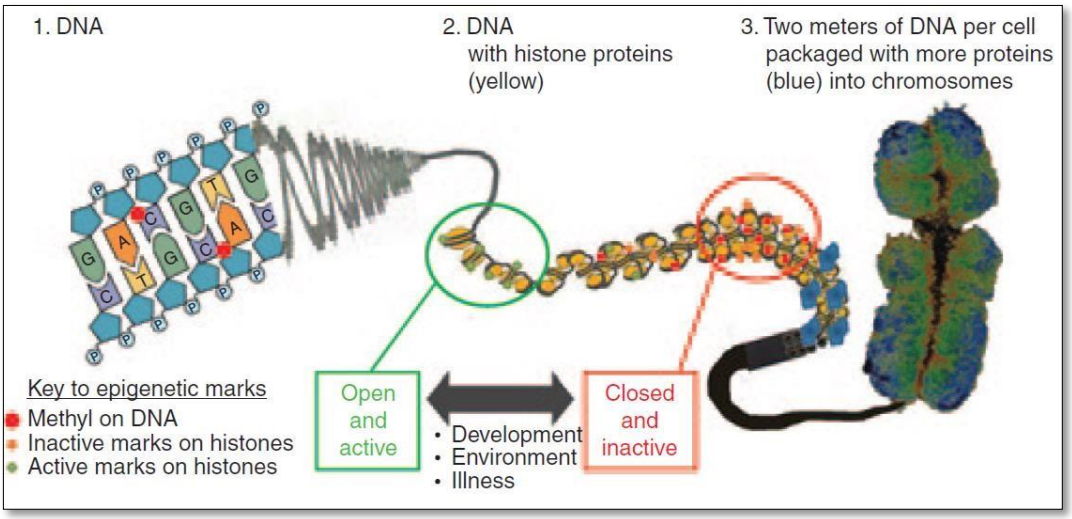


Figure 10. *Main mechanisms at the basis of epigenetics: covalent modification of the chromatin and noncoding RNA. The principal chromatin modifications are mostly in CpG dinucleotide configurations, and posttranslational modifications including methylation and acetylation of the histone proteins forming the nucleosome. These modifications are set-up or removed by the enzymes of the epigenetic machinery, leading to a dynamic state of the chromatin. The combination of all epigenetic marks over the genome constitutes the epigenome. The epigenetic landscapes are transmitted through cell division, leading to a memory of the cell identity, which leads to the notion of their 'heritability'.*

Because of its dynamic nature epigenetic can be sensitive to the environment. In fact it has been demonstrated a link between nutrition, energy metabolism and epigenetic processes[16].

i. Blueberry Bioactive Compounds

Green leafy vegetables and blueberries have been demonstrated to have important antioxidant properties thanks to polyphenols and phenolic bioactive compounds such as anthociyanines[17].

Both anthocyanins and proanthocyanins have been shown to reduce inflammation via the NF-kB and TNF-α pathways[18,19]. Other studies have established that blueberries attenuate bacterial translocation to extraintestinal sites, a phenomenon implicated in the inflammatory and metabolic pathology associated with diets high in fat[20] and that they inhibit macrophage infiltration in adipose tissue, reduce their activation and consequently reduce adipocyte death with the final result of improving significantly insulin sensitivity[21,22].

In addition phenolic extracts have been demonstrated to be capable to reduce production of nitric oxide and prostaglandin E-2, the expression of inducible nitric oxide synthase and cyclooxygenase in lipopolysaccharide-stimulated RAW 264.7 macrophage[23].

More recently it has been proved that blueberry anthocyanins are able to increase the total antioxidant capacity of the retina in diabetes subjects by reducing VEGF and IL-1β levels and by enhancing the activation of Nrf2/HO1 signalling, an important modulator of cell death and proliferation[24].

A reduction in inflammatory status by anti-inflammatory agents could constitute a potential approach to reduce adverse obesityassociated consequences.

Low consumption of fruit and vegetables is common throughout the world[25]; because of its feasibility a dietary supplementation with bioactive components in blueberries is an attractive dietary intervention in order to increase and maintain an individual's fruit and vegetables consumption over a long period of time[26].

2. Our approach and study design

Considering these dogmas and considering the elevated therapeutic frustration in achieving results with overweight and obese individuals, it was our goal to consider an efficacious alternative way to prevent childhood adiposity.

The project's overall objective was to examine the role of dietary supplementation with bioactive in freeze-dried whole blueberry powder on pro-inflammatory (**TNF-α**, **IFN-γ**, **Leptin, C-reactive protein**) and anti-inflammatory (**IL-4** and **TGF-β**) adipokines and cytokines in nulliparous overweight or obese pregnant women and on birth weight and at age one.

We hypothesized that increased daily consumption of blueberry bioactive, based on pre-clinical data, would be effective in reducing pro-inflammatory and in increasing anti-inflammatory adipokines and cytokines therefore reducing systemic inflammation and ultimately result in reducing new-born birth weight and at age one.

The study design is a controlled clinical trial.

3. Materials and Methods

a. Study population

This study is part of the "S3PIO Program" (Swiss Project for Precocious Prevention of Infantile Obesity), a Swiss community base cohort in Canton Ticino.

Approval for the study was obtained by Ethic Commission of the Ente Ospedaliero Cantonale (EOC). All woman provided written informed consent. Parents or caretaker provided written informed consent for the health check at one year of age.

i. Phase 1

The *Phase 1* of the study was conducted between January 2014 and December 2014. All nulliparous pregnant woman (starting number = 900) with a BMI (calculated as weight (kg)/[height(m)]^2) more than 25 (overweight/obese and severely obese), starting from 13 weeks of gestation; (IQR, 12-14 weeks; n= 117; 13,3%) that underwent a first Gynaecological and Obstetrical visit were invited to participate in the study. 15 women denied their consent. 102 women agreed to complete the study and to enter in the Control group.

ii. Phase 2

The *Phase 2* of the study was conducted between January 2016 and December 2016. All nulliparous pregnant woman (starting number = 872) with a BMI (calculated as weight (kg)/[height(m)]^2) more than 25 (overweight/obese and severely obese), starting from 13 weeks of gestation; (IQR, 12-14 weeks; n= 107; 12,2%) that underwent a first Gynaecological and Obstetrical visit were invited to participate in the study. 2 women denied their consent. 105 women agreed to complete the study and to enter in the Blueberry group.

All woman were asked to attend a periodic monthly diet and medical visit.

Women who gave birth to twins, delivered preterm (< 37 weeks of gestation), with history of psychiatric disease prohibiting adherence to study protocol, history of allergic reactions to blueberries, consuming berries, grapes and wine more than 3 times a week, with diabetes or consuming lipid-altering medication (e.g. thyroid hormones, steroids, sleep medications, insulin use and epileptic drugs), and actively smoking, were excluded.

Birth outcomes (gestational age at birth, sex, birth weight and congenital abnormalities) were obtained; new-born with suspected or confirmed congenital abnormalities were excluded.

After the child first birthday a health check was done. Children with diagnosis of genetic obesity or that underwent steroid therapy or assumed other appetite-enhancing drugs (anti-histamine and steroids)

for a prolonged period of time (at least 7 or more days) were ruled out (see **Figure 10** for specific details of the study population).

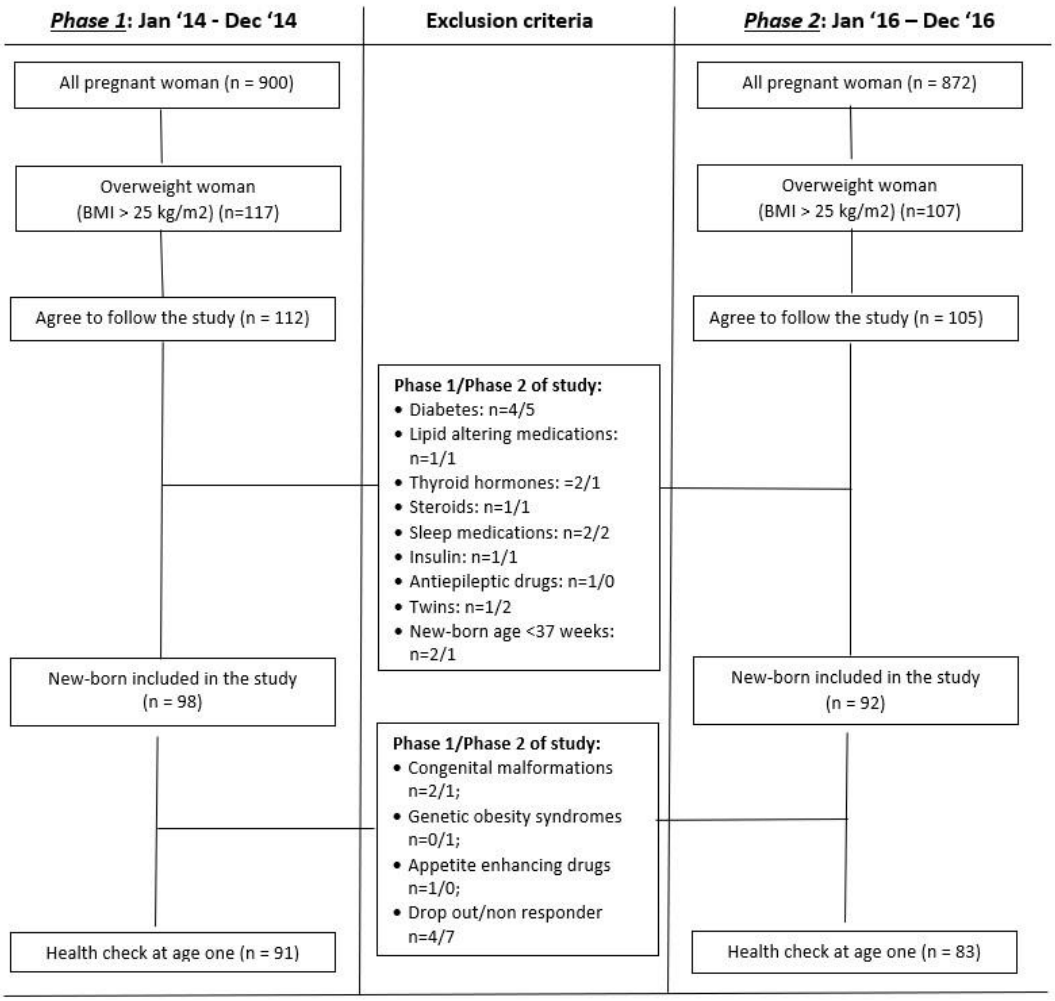


***Figure 11**. Flow chart of the study population*

b. Clinical intervention and source of whole blueberry bioactive

A freeze dried whole blueberry powder was prepared by the United States Highbush blueberry Council. The whole blueberry powder was made from a mixture of 50/50% of 2 varieties of highbush blueberries, Rubel (*Vaccinium corymbosum*) and Tifblue (*Vaccinium ashei*).

The whole blueberries were freeze-dried, milled, and stored in aluminium cans under nitrogen. The 45 g of blueberry powder contained 1462 mg of total phenolics, 668 mg of anthocyanins, and 16.02 mmol TE of antioxidants (oxygen radical absorbance capacity). Also, the 45 g of blueberry powder that was provided to the participants equated to an amount of bioactive in $\sim$2 cups of fresh whole blueberries.

All pregnant women of Phase 2 of the study (n = 105) agreed to receive twice daily a smoothie with blueberry bioactive added during the last trimester of pregnancy (starting from the 28th week of gestation).

The participants were instructed to consume 1 smoothie at breakfast meals and the other smoothie at dinner meals (at least 6 h apart). The smoothies were prepared in the metabolic kitchen and a supply of frozen smoothies was provided in a cooler for the participants to pick up at each monthly visit.

Participants were instructed to keep the smoothies frozen, thaw them in the refrigerator, avoid exposing them to direct heat, and avoid adding any other ingredients to them.

For study compliance, the participants verbally reported their smoothie consumption to the dietitian at each visit. A compliance of >75% was mandatory for continued participation in the study.

c. Food records and questionnaire

At the screening visits a registered dietitian instructed participants to record a detailed food record. Participants were asked to provide labels and/or recipes for accuracy of the food records. The dietitian reviewed the food records for accuracy and completeness.

Based on their eating patterns and usual intake, participants were counselled by the dietitian on ways to remove ≈2000 kj/d (500 kcal/d) from their daily intake to compensate for the energy consumed in the blueberry and placebo smoothies.

Participant were asked to maintain their current body weight and physical activity or they would be extruded from the study. The participants body weight were measured monthly to monitor weight maintenance. A change of ≥ 1 kg of body weight was addressed by the dietitian and proper counselling was provided. They also reported adverse events and changes in medication during the study.

Smoothie rating and fruit/wine questionnaires were also used in the study. Before starting the study, participants were given the opportunity to taste the smoothie for acceptability.

The fruit/wine questionnaire was administered at each visit as a reminder to abstain from berries, grapes, juices that contained berries and grapes, and wine throughout the study. The rationale for these questionnaires was to eliminate consumption of anthocyanincontaining foods and drinks.

d. Clinical evaluation

All pregnant women underwent a complete auxological evaluation. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2) and mothers were classified as normal (< 25 kg/m^2), overweight (≥ 25 kg/m^2) according to the World Health Organisation criteria.

The clinical evaluation of the new-born was conducted at day 1 and included a general visit for detection of any sign of disease.

Length and weight were measured using a Marsden M-400-80D Baby Scale with Height Rod. Head circumference was measured using a SECA measuring tape from the most prominent part of the forehead (often 1-2 fingers above the eyebrow) around to the widest part of the back of the head.

A large for gestational age new-born was classified using the WHO Child growth standards based on length/height, weight and age[27].

The clinical evaluation of children at age one included height, weight and waist circumference.

Height was determined using a Leicester portable height measure (SECA, Hamburg, Germany) and weight using a Marsden MS4102 weighing scale (Oxfordshire, United Kingdom). Waist circumference was measured midway between the coastal border and the iliac crest to the nearest millimetre using a SECA measuring tape.

Normal, overweight or obese children were classified using the WHO Child growth standards based on length/height, weight and age.

A condition of overweight and obesity at age one was defined for BMI z-scores over 97th percentile and over 99th percentile on specific children BMI scale, respectively.

e. Biochemical assays

Blood samples were obtained from healthy pregnant women at 36th week of gestation during a healthy regular gynaecological check; women with recent (one week) inflammatory disease were excluded from the study. Serum was separated and kept frozen at -70°C until analysis. The subsequent variables were studied:

i. Proinflammatory adipocytokines

Serum levels of cytokines TNF-α were measured using commercial available ELIZA kits (Ani Biotech Orgenium Laboratories Business Unit, Finland) Bender MedSystems GmbH Campus Vienna biocentre and the sensitivity of detection for TNF-α was 2.3 pg/mL; Leptin levels were measured with an ELIZA procedure using commercial kits (Diagnostic Biochem Canada Inc. 1020 Hargrieve Road) and the sensitivity of detection level was 0.5 ng/mL. Serum levels of IFN-γ were analysed using ELISA method (R&D Systems, Wiesbaden, Germany) and the sensitivity of detection was 8 pg/mL.

ii. Anti-inflammatory adipocytokines

TGF-β was measured using ELISA method (R&D Systems, Wiesbaden, Germany) and the sensitivity of detection was 15.4 pg/mL. Serum IL-4 levels were determined with an enzyme-linked immunosorbent assay (ELIZA) technique using commercial kits

(Orgenium Laboratories, FIN-00790, Helsinki, Finland) and the sensitivity of detection was <7 pg/ml.

iii. Indicator of systemic inflammation

Serum samples were analysed for high sensitivity CRP level by turbidimetry (ADVIA 2400, Siemens Healthcare GmbH, Erlangen, Germany).

f. Statistical analysis

Analyses were performed using GraphPad Prism version 7. Differences between the blueberry and control group were analysed by a 2 sample t-test (continuous data) and within groups analysed by a paired t-test. P values ≤ .05 were considered statistically significant. Data were expressed as means ± SEM.

4. Results

a. Study population

i. Pregnant women

112 women entered in the control group and 105 in the blueberry treated group. The two population were age-matched and did not differ in education. Furthermore the two groups did not differ in body weight or adiposity and in energy and macronutrient consumption (protein, carbohydrate, and fat; data not shown). In both groups the percentage of caesarean section (programmed and/or urgent) was similar (see **Table 2**).

ii. New-born

Of all new-born, 98 patients entered in the control group and 92 in the blueberry group. A slight prevalence of female was seen in the control group (53 vs 51%, respectively). 7% of the new-born in the control group were defined as large for gestational age (LGA). No LGA infant was detected in the blueberry group. Gestational age was similar (see **Table 2**).

iii. Childhood

At age one, 91 patients in the control group and 83 in the blueberry group were studied. Exclusive breastfeeding for 6 months was registered in similar percentage in the two groups. In the control group, 18% of the children were overweight and 7% were obese. In the blueberry group 13% of the children were overweight and 7% were obese (see **Table 2**).

Variables	**Control**	**Blueberry**
Pregnancy:		
Race *(Caucasian/other*), n/n	87/25	80/25
Age, *y*	29 ± 3	30 ± 2
Education**:**		
After primary school, n/n	62/112	58/105
Overweight, n/n	73/112	77/105
Obese, n/n	39/112	28/105
Median BMI, kg/m^2	28,98 ± 0,29	28,2 ± 0,28
Median BMI in overweight, kg/m^2	27,24 ± 0,2	26,71 ± 0,15
Median BMI in obese, kg/m^2	32,55 ± 0,27	32,3 ± 0,36
Caesarean section, n/n	30/112	29/105
Birth:		
Sex, *m/f*	46/52	45/47
Gestational age, *days*	273 ± 8	275 ±5
LGA, n/n	7/98	0/92

Offspring (age one):		
Sex, *m/f*	43/48	42/41
Breastfeeding for 6 months, *n/n*	50/91	46/83
Overweight, n/n	16/91	11/83
Obese, n/n	6/91	6/83

Table 2: *anthropometrics and other variables of examined pregnant women, new-born and children in control group and in the blueberry treated group *.*

**Values are means ± SEM.*

b. Adipocytokines

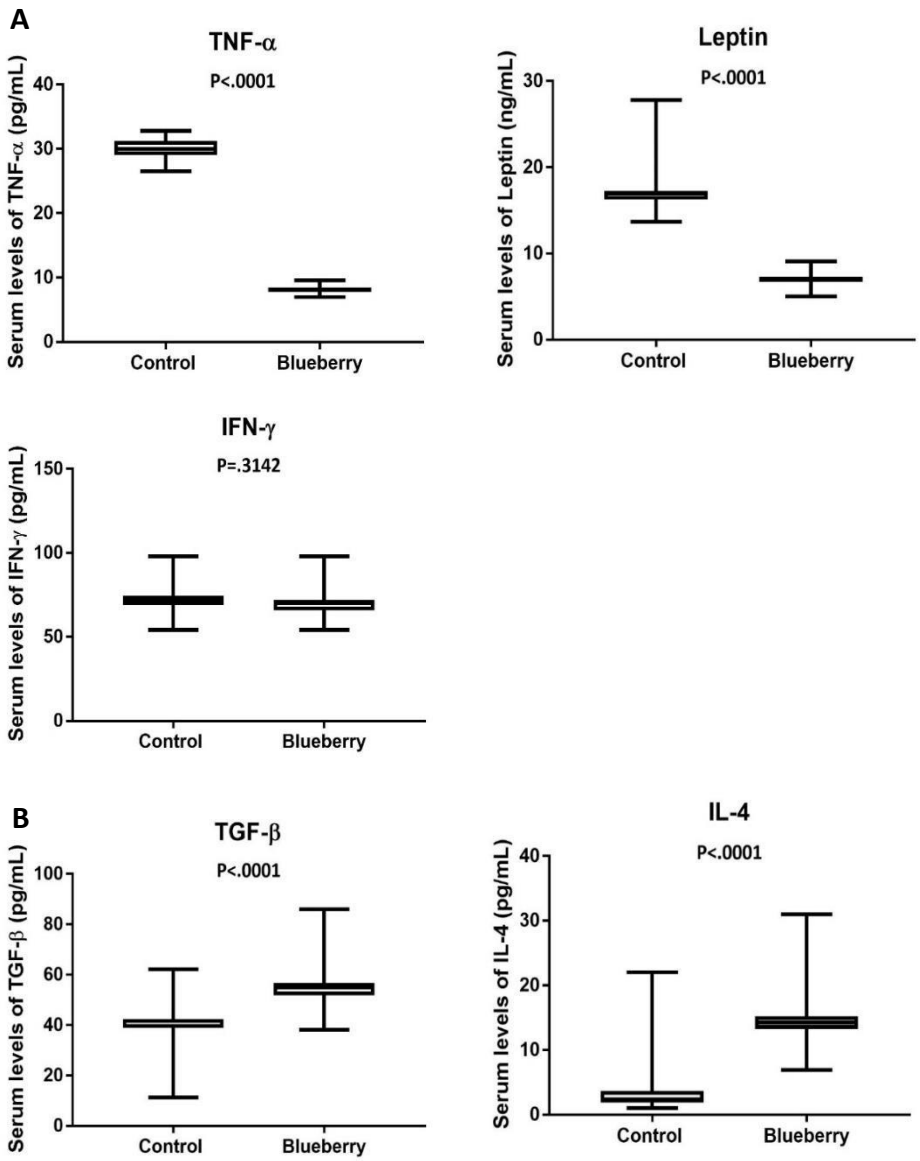
Blood samples was tested in 27 age and weight-matchedhealthy pregnant women in the control group and in 27 pregnant women in the blueberry group (after a treatment of 8 week with blueberry bioactive).

Levels of pro-inflammatory cytokines (TNF-α; Leptin) and CRP were significantly lower in the blueberry group. Parallelly levels of antiinflammatory cytokines were significantly higher in the blueberry group. No difference was seen for levels of IFN-γ between the two groups (see **Table 3** and **Figure 11** for details).

Variables	**Control**	**Blueberry**	**Significance**
Cytokines:			
Pro-inflammatory			
TNF-α (pg/mL)	29,89 ± 0,283, n=27	8,124 ± 0,1085, n=27	P <.0001
Leptin (ng/mL)	17,28 ± 0,4742, n=27	6,977 ± 0,1187, n=27	P <.0001
IFN-γ (pg/mL)	72,77 ± 1,522, n=27	70,46 ± 1,684, n=27	P = .31
Anti-inflammatory			
IL-4 (pg/mL)	4,196 ± 0,9752, n=27	14,87 ± 0,8844, n=27	P <.0001
TGF-β (pg/mL)	14,87 ± 0,8844, n=27	55,22 ± 1,47, n=27	P <.0001
C-Reactive Protein (mg/L):	5,917 ± 0,698, n=27	3,741 ± 0,6279, n=27	P = .024

Table 3: *comparison between pro-inflammatory, anti-inflammatory cytokines and adipokines and C-reactive protein levels in overweight and obese pregnant women in control and in the blueberry group*.*

**Values are means ± SEM.*



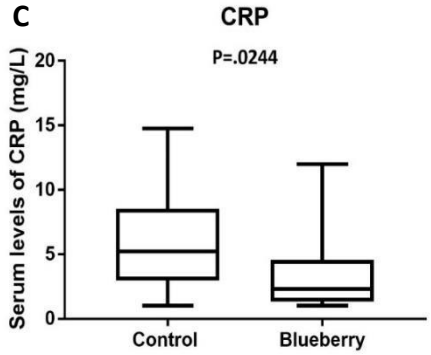


***Figure 12**: Comparison between pro - and anti -inflammatory cytokines levels in overweight and obese pregnant women in control and in the blueberry group. **A**. Mean change in IFN -γ; TNF-α and Leptin levels between control and blueberry group. A significative lower level of inflammatory cytokines was seen in the blueberry group according to TNF-α and Leptin; no difference was seen for levels of IFN-γ. **B.** Mean change in TGF-β and IL-4 levels between control and blueberry group. A significative higher level of anti-inflammatory cytokines was seen after treatment with blueberry. **C**: Levels of C -reactive proteins, marker of systemic inflammation, in control and blueberry group. Notice the significative lower CRP levels in the blueberry group.*

c. Birth weight

Birth weight improved significantly in the blueberry group. In particular male and female birth weight reduced significantly (male: 3636 gr ± 56,7 in control group and 3302 gr ± 60,9 in the blueberry, P=.001; female: 3446 gr ± 53 in control group and 3094 gr ± 65,5 in the blueberry, P<.0001), see **Figure 13** for details.

d. Weight at age one

At one year of age weight continues to remain significantly lower in the blueberry group. Precisely male weight reduced significantly in the blueberry group (control: 9597 gr ± 134,4; blueberry: 8656 gr ± 271,9; P=.0021) and the same happened in females (control: 8812 ± 167,5; blueberry: 8083 ± 266,3; P=.0211); see **Figure 13** for details.

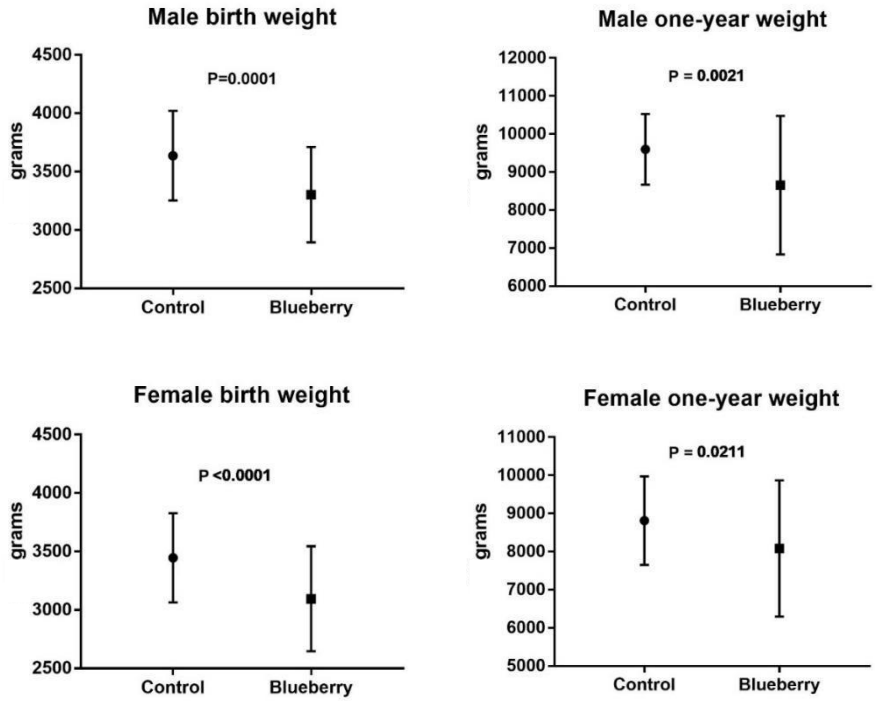


Figure 13: *Mean change in birth weight and weight at age one in male and female whose mothers consumed or not the blueberry smoothie. Values are means ± SEM, male: n=46 (control) or 45 (blueberry) at birth and n=48 (control) or 45 (blueberry) at age one; female: n=52 (control) or 47 (blueberry) at birth and n=48 (control) or 45 (blueberry) at age one.*

5. Discussion

To our knowledge this is the first controlled clinical trial that evaluates the effect of a daily blueberry consumption in overweight pregnant women and specifically on their cytokine milieu.

The two population studied, although tested in different period, were identical in physical appearance and macronutrient content with the exception of adding the blueberry bioactive to the blueberry smoothies.

Our dietitian worked with the participants during the monthly visits to eliminate its calories from their diets to compensate for the energy provided by the smoothies and the constant supervision of our medical doctor and dietitian helped our population in maintaining a stable body weight and physical activity throughout the whole study without making them potential confounding factors.

Breastfeeding period and time of weaning were similar in the two population ruling them out as other potential confounders.

Consumption of smoothies has been demonstrated to be an attractive and convenient dietary approach for those adults who do not consume the recommended daily amounts of fruits and vegetables. Our study population appreciated this form of integration: the compliance reached 92% (data not shown).

Persistent data's have clearly linked inflammation to adiposity with significant report on the mechanisms by which inflammation at whole body level attenuates insulin action[28] that is consequently secreted in higher quantities.

Overweight women tends to have hyperglycaemia that associated with higher levels of inflammation leads to foetal hyperinsulinemia, increased utilization of glucose and, hence, increased foetal adipose tissue and foetal macrosomia (modified Pedersen's Hypothesis, see **Figure 14** for details)[29].

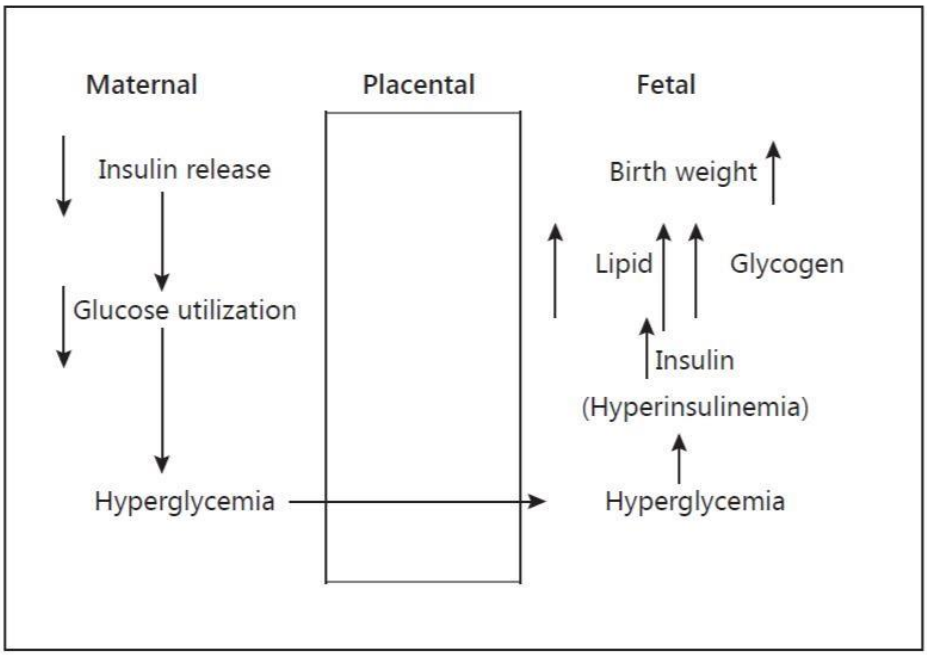


***Figure 14**: Results of maternal hyperglycaemia modified according to Pedersen's hypothesis*

Blueberry bio-active compounds have been demonstrated to endow antioxidant properties (radical scavenging)[30] and to improve insulin sensitivity in obese, non-diabetic and insulin resistant

individuals[26]. Vuong T et al. found that 6-hours treatment of fermented blueberry juice with and without insulin increased glucose uptake into the muscle and adipocyte cells[31].

Contrary to what was seen by Stull J et al, and according to DeFuria et al, in our case a consumption of daily doses of bioactive in blueberries, is capable in altering the inflammatory biomarker profile and in particular it significantly reduces levels of TNF-α, a proinflammatory cytokine that exerts numerous effects in adipose tissue and that reduces anti-inflammatory cytokines like adiponectin and of Leptin, an inflammatory adipokine secreted primarily by fat cells ant that acts centrally particularly in the hypothalamus, is reduced.

Curiously and parallelly we discovered that IL-4, a cytokine that inhibits lipid accumulation in fat tissues, is significantly elevated in the blueberry group as well as TGF-β, a well-known cytokine with immunosuppressive and healing properties[32], that is significative increased after blueberry consumption.

Therefore one of the major findings of our study is that blueberry bioactive are capable to reduce the inflammatory status by inducing a switch to a more tolerogenic cytokine milieu. As a result insulin sensitivity is increased, levels of insulin are reduced and protein and fat stores are consequently reduced in the foetus (see **Figure 15**).

The final confirmation of these properties is demonstrated by the significative reduction of a systemic marker of inflammation such as the CRP.

Another major finding of our study was that a daily integration of whole blueberry bioactive reduced significantly birth weight (parallelly the risk of delivering an LGA infant) and consequently the risk of serious adverse pregnancy outcome. More interestingly this result is maintained in the offspring because weight at age one was still significantly reduced in mothers who consumed blueberry extracts.

Our study gives us a possible confirmation that epigenetic has a dynamic nature and that nutrients and their metabolites can be direct activators or inhibitors of the epigenetic machinery enzymes[33].

The persistence of this beneficial effect on children at age one could be a direct effect of an altered epigenetic machinery enzymes or

a direct effect on the foetus metabolic ambient; in fact flavonoids are able to pass the placenta[34] and therefore able to exert their antioxidant properties directly on the foetus.

Further studies are needed in this field in order to better understand this interesting result.

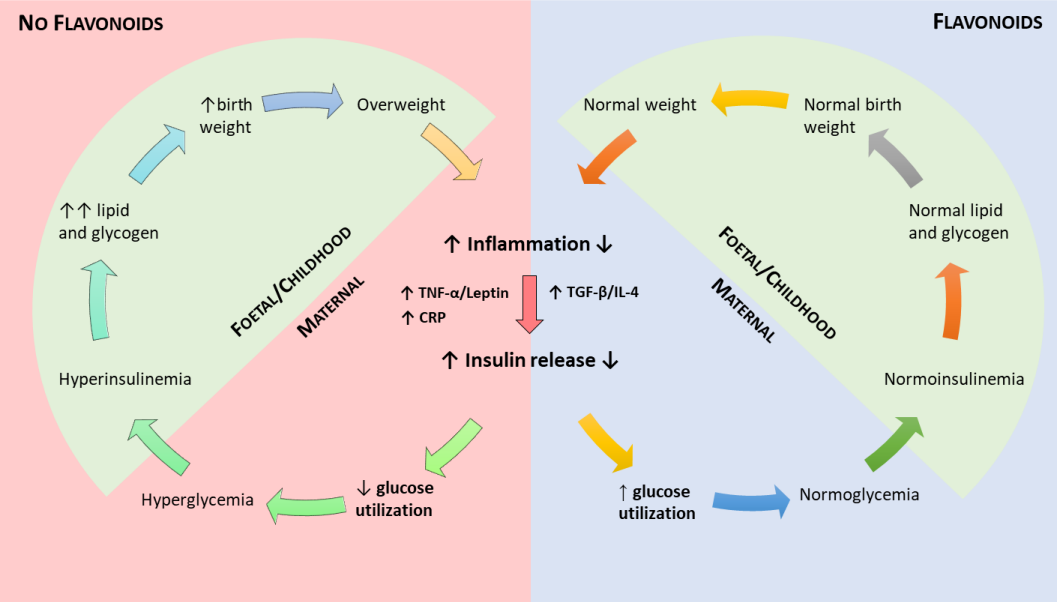


***Figure 15**: Beneficial effects of blueberry consumption in overweight pregnant women; Its bioactive compounds are able to reduce inflammation by inducing tolerogenic cytokines and consequently reduce insulin release an ameliorate glucose utilization with the final effect of a reduced fat and protein deposition in the foetus and therefore reduced birth weight and weight at age one.*

6. Conclusion

In conclusion we can affirm that a 12 week daily supplementation of bioactive in freeze-dried whole blueberry powder in the last trimester of pregnancy of overweight pregnant women, in association with a regular dietary surveillance, is able to reduce the inflammatory cytokine milieu hence inducing a better insulin sensitivity with the final result of reducing birth weight and at age one.

Considering its feasibility this could be an efficacious way to prevent childhood obesity.

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