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Higher protein intake increases cardiac function parameters in healthy children. Metabolic programming by infant nutrition: secondary analysis from a clinical trial

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ABSTRACT

Background: Protein intake may modulate cardiac structure and function in pathological conditions, but there is a lack of knowledge on potential effects in healthy infants.

Methods: Secondary analysis of an ongoing randomized clinical trial comparing two groups of infants receiving a higher (HP) or lower (LP) protein content formula in the first year of life, and compared to an observational group of breast-fed (BF) infants. Growth and dietary intake were assessed periodically from birth to 2 years. Insulin-like growth factor 1 (IGF-1) axis parameters were analyzed at 6 months in a blood sample. At 2 years cardiac mass and function were assessed by echocardiography.

Results: HP infants (n=50) showed a higher body mass index z-score at 2 years compared to LP (n=47) or BF (n=44). Cardiac function parameters were increased in the HP group compared to the LP, and were directly related to the protein intake during the first 6 months of life. Moreover, there was an increase in free IGF-1 in the HP group at 6 months.

Conclusions: A moderate increase in protein supply during the first months of life is associated with higher cardiac function parameters at 2 years. IGF-1 axis modifications may, at least in part, underlie these effects.

INTRODUCTION

There is compelling evidence that the type of nutritional exposure in early periods of an organism's development may affect the structure and function of body tissues later in life (1,2). Protein intake may play a key role modulating body mass and function of various organs and systems (3,4). This influence can be exerted by a direct effect on the target organs (5) and via the modulation of whole body size (6) resulting in functional load of vital organs. Recent evidence indicates that feeding infant formulas with higher protein content resulted in an increased kidney volume, with a direct effect on kidney function, and an increase in whole body mass (3,7). One of the target organs affected by early protein intakes is the heart, the development of which occurs in an early embryonic phase. As such, early post-natal nutrition would affect growth since this is the period in which the fastest growth of the body and organs take place. Effects of protein supply in pregnancy on left ventricular function have been described in rats (8). Similarly, infants and children suffering from protein-energy malnutrition have been shown to have reduced left ventricular mass and function (9–11) proportional to body surface (10). In animal models, early-life over-nutrition has been associated with left ventricular hypertrophy (12). Investigations have been conducted on cardiac function parameters under extreme protein supply situations, but data are lacking on the effect of normal protein supplies on these parameters.

Proteins serve as structural substrates for heart tissue development. However, proteins can also affect the growth of the heart through a systemic stimulation of the constituents of the IGF-1 axis. The rationale in suggesting this mechanism is based on the observation that increased protein intakes, via infant formula diets, under non-extreme conditions, stimulate the growth of other organs, such as the kidney (7). Further, cardiac hypertrophy in acromegaly is partly mediated by the GH/IGF-1 axis; a meta-analysis

confirmed that treating acromegaly with IGF-1 analogues (aiming to reduce the GH/IGF-1 axis activity) reduces the cardiac hypertrophy and improves hemodynamic parameters (13). These findings in adults were also observed in younger patients as well as in animal models (14–16). Recently, we found that non extremely variations in protein supply with infant formulae resulted in varying kidney growth and function in healthy infants, and this was partly mediated by modulation of the IGF-1 axis (3,7). The aim of the present study was to assess the effect of different protein intakes during the first year of life on cardiac mass and function at 2 years of age in healthy infants.

RESULTS

Study sample

Of the 261 Spanish children remaining in the original EU-CHOP randomized trial (trial number in clinicaltrials.gov NCT00338689), 145 accepted the invitation to attend an additional visit to perform the cardiac assessments at the age of 2 years. The assessments were performed between November 2004 and March 2006. The different study groups were composed of 100 formula fed children of whom 51 and 49 had Higher Protein (HP) and Lower Protein (LP) formula, respectively, and 45 were Breast Fed (BF). Technically acceptable echocardiographic assessments were obtained in 141 children (97% of the participants); in 4 cases, the triplicate measurements were unsatisfactory, and were discarded from the statistical analyses. Triplicate blood pressure measurements were obtained in 110 children (78% of the participants); 31 children did not fulfill the criteria of technically-acceptable readings. At 6 months of age a blood sample was drawn in 109 children from the 141 who had had valid echocardiography (Figure 1). All available data from each output were used for the analyses. There were no significant differences in anthropometric measurements from

birth to 2 years between infants who had the cardiac assessment and those who did not. Gender distribution as well as anthropometry at birth (weight, length and head circumference) did not differ between the 2 formula-fed groups, or between the formula and breastfed groups.

Effect of protein intake on anthropometry

As previously observed in the whole EU-CHOP cohort (6), the HP group had higher BMI z-scores compared to the LP group (0.68 ± 0.77 *vs.* 0.22 ± 0.84, p=0.005 for HP and LP, respectively) at 2 years of age. In addition to the BMI z-score, we found significant differences between groups in terms of absolute BMI (16.7 ± 1.0 *vs.* 16.0 ± 1.1, p=0.006 for HP and LP, respectively) whereas weight and height did not differ significantly between groups; neither as absolute values (weight: 12.7 ± 1.3 *vs.* 12.3 ± 1.2, p= 0.173; height: 87.4 ± 3.2 *vs.* 87.8 ± 2.7, p=0.445, for HP and LP respectively) nor standardized as z-score.

Cardiovascular parameters

Results from the echocardiographic assessment showed that cardiac function parameters were modulated by protein intake, whereas cardiac mass didn't differ between the feeding groups. LVM was slightly higher in the HP group, but this difference did not reach statistical significance (29.5 ± 5.6 *vs.* 29.13± 4.3, p=0.69 for HP and LP, respectively). Similar results were observed with respect to the LVMI (42.6 ± 7.7 *vs.* 41.4 ± 6.0, p=0.400 for HP and LP, respectively). However, both EF and SF were higher among children receiving the HP formula compared to the LP group (69.1 ± 5.1 *vs*. 66.7 ± 4.9 for EF and 37.7 ± 4.1 *vs.* 35.8 ± 3.8 for SF, p<0.05 for both groups) (Figure 2). Linear regression models using the protein intake (g/day) from the monthly

food diaries were applied to estimate the effect of the formulas on cardiac function. Results showed that each additional g/day of protein intake during the first 6 months of life increased EF and SF at 2 years (Figure 3, panel A). The effect of protein increase was greater during the first months and decreased progressively subsequently; EF increased by 0.52% for each g/day of protein intake increase during the 1st month of life ($p<0.001$), 0.43% during the 2nd month of life ($p=0.001$), 0.31% during the 3rd ($p<0.01$) and 0.17% during the 6th month ($p<0.05$). The EF and SF values at 2 years were independent of the protein intake from the 6th month onwards. Similarly, protein intake during the first 6 months (but not subsequently) showed a significant effect on weight and BMI at 2 years (Figure 3, panel B). Multivariate linear regression models adjusted for anthropometric variables were applied to assess whether there was a direct effect of type of formula feeding on EF. Results showed that HP formula feeding was significantly associated with an increase of 2.03 (95%CI: 0.07, 4.00) units ($p=0.043$) in EF, despite adjusting for weight z-score. Both variables explained up to 11.3% of EF variability while weight z-score alone explained 8.6%. Results of these analyses were similar for SF (data not shown).

Cardiovascular variables were associated with anthropometry at 2 years (Table 1). Cardiac mass and function were significantly correlated with BMI z-score, weight z-score, length z-score, weight gain per month z-score, and length gain per month z-score. Relationships between cardiac mass and anthropometry values disappear when LVM was indexed as LVMI. In our study we did not find significant relationships between anthropometry and blood pressure measurements (neither with systolic nor diastolic pressure). We predicted the energy expenditure for each child using the individual's anthropometric data. This parameter was directly correlated with EF ($r = 0.30$,

p<0.001), SF (r = 0.32, p<0.001) as well as with LVM (r = 0.43, p<0.001). Values of energy expenditure differed between genders (1013 ± 49 *vs.* 942 ± 72 kcal/day for boys and girls respectively, p<0.001) but were similar for the overall study groups (HP: 977 ± 78; LP: 982 ± 70; BF: 966 ± 67 kcal/day; p=0.578).

Protein intake and IGF-1 axis

Results of the IGF-1 axis parameters segregated with respect to feeding groups are depicted in Figure 4. HP-fed children showed higher concentrations of free IGF-1, compared with the LP and BF groups and lower concentrations of IGF-1 binding protein 2 (IGFBP2) compared to LP and BF groups (p<0.001). Total IGF-1 and IGF-1 binding protein 3 (IGFBP3) concentrations were higher in both formula-fed groups compared to BF children.

Relationship between IGF-1 axis, anthropometry and cardiovascular variables

Total and free IGF-1 showed linear associations with weight z-score and BMI z-score at 6 months (total IGF-1: r = 0.300 and r = 0.255, p<0.01; free IGF-1: r = 0.330, p<0.001 and r = 0.324, p<0.01 for weight z-score and BMI z-score, respectively). Also, free IGF-1 was correlated with weight z-score (r = 0.242, p=0.011) and BMI z-score (r = 0.204, p=0.032) at 2 years.

No statistically significant relationships were found between cardiac mass at 2 years and the IGF-1 axis parameters at 6 months. Conversely, cardiac function parameters were associated with IGFBP2 (r = -0.199 and r = -0.201, p<0.05 for EF and SF, respectively) and blood pressure at 2 years was associated with free IGF-1 (r = 0.303, p=0.01 and r = 0.389, p<0.001 for SBP and DBP, respectively) measured at 6 months of age.

Finally, multivariate analyses with linear regression models measuring the influence of

feeding type on cardiac function parameters adjusted for weight z-score were not altered when IGF-1 was introduced into the model (data not shown).

DISCUSSION

Nutritional factors influence subsequent development of cardiac function (17,18). Such relationships have only been documented in situations where one or the other was grossly altered, i.e. malnutrition or cardiac pathology. Our study explores, for the first time, the association between neonate nutrition and cardiac function in healthy infants. Our results show that a higher protein intake in infancy is associated with a higher cardiac systolic function at 2 years of age.

Effect of protein intake on body structure and cardiovascular parameters

Protein intake during infancy is one of the key nutritional factors affecting obesity risk, and other health consequences later-in-life (1,2,6). In concordance with the overall EU-CHOP findings, our results in this (Spanish) subsample support the hypothesis that a higher protein intake during the 1st year of life (but within the EU recommended range), produces an increase in the subsequent BMI z-score (measured at 2 years), which may confer an increase in subsequent obesity risk (19).

Nutritional factors are able to modulate cardiac function parameters. In animal models, exposure to an excess lipid intake can worsen heart remodeling, leading to contractile dysfunction (20) while, in elderly people, amino acid supplementation improved EF (18). However, the effects of variation in protein intake on cardiac function in healthy neonates have not been studied. Our findings indicate that an increase in protein intake during the 1st year of life was associated with a subsequent increase in cardiac function parameters measured at 2 years of age. Specifically, the association with cardiac

function was found with protein intake during the first months of life, rather than the intake closer to the time of cardiac function measurement. That such an effect of protein intake can be observed one year after the end of the intervention, would suggest a programming effect. As expected, we also observed a direct relationship between anthropometric variables and cardiac function parameters. Therefore, one could hypothesize that the effect of protein intake on cardiac function could be exerted indirectly through an anthropometric modulation of body size. This hypothesis would be in line with the proposed mechanism by which an increase in total body surface implies an increase in the cardiac output to fulfill the organism's higher O_2 requirements (21). Higher body weight implies higher energy expenditure, and energy expenditure is tightly related to cardiac output. Accordingly, as in our study, protein intake would be associated to weight gain (6,22,23) and, in which, weight gain as well as protein intake would be related to cardiac function. On the other hand, anthropometric measures were associated with energy expenditure and cardiac function. This suggests that an increase in body surface would be accompanied by a rise in cardiac output which, in turn, depends directly on the EF. Despite the evidence of an indirect effect of protein intake on cardiac function parameters via body size modulation, a direct effect on the cardiac system cannot be completely rejected since we had found that the type of formula was significantly associated with EF, even in the multiple regression models when adjusted for body size.

Cardiac mass has been shown to vary with dietary protein intake in pathologic situations such as protein-related malnutrition in humans (24) or overfeeding in rats (12). In our study, the effect of early protein intake on the body-growth parameters was accompanied by the expected association of these parameters with the LVM (21,25). One of our objectives was to determine whether the protein supplied by milk-formula

during fast-growth periods could induce changes in cardiac structure, as evidence by changes in cardiac mass measured by echocardiography. We observed a slight increase in the LVM among those children receiving the HP formula during the 1st year of life, but these differences did not reach statistical significance. The lack of significance could be because of the limited sample size precluding the identification of subtle changes. The short time-span over which the study was conducted may have contributed, as well. Our results suggest that an effect of protein intake on cardiac mass may be produced via modulation of body size and structure. The rationale for this conclusion was the direct correlation between anthropometric variables and LVM, but not with LVM, as indexed by length.

A possible protective effect of breastfeeding on later development of hypertension, secondary to a lower protein intake with human milk, has been proposed (26). In our study we did not find significant differences in blood pressure measurements between the feeding groups, which may be influenced by the young age of the participants at the time of the cardiac study.

Role of the IGF-1 axis

Protein intake has been directly associated with IGF-1 secretion (27,28). This mechanism is partly responsible for the protein-induced increase in anthropometric parameters, as well as in some internal organs such as the kidney in healthy children (7,29). Protein intake induces IGF-1 secretion which, in turn, induces an increase in weight through its anabolic and metabolic effects in most cell lines (30,31). As cardiac growth and remodeling are also influenced by the IGF-1 in a physiologic as well as pathologic manner (32–34), we sought relationships between these parameters. Our findings show that the HP group presented higher concentrations of free IGF-1 as well

as lower concentrations of IGFBP2 (transport protein that inhibits the IGF-1 action) compared to LP and BF groups. IGF-1 concentrations were associated with weight and BMI at 6 months, while free IGF-1 also correlated with these parameters at the age of 2 years. However, we did not find any relationship between IGF-1 axis parameters (neither total nor free IGF-1, nor its transport proteins) with cardiac mass.

. The protein-induced increase in weight and size of certain organs produces a metabolic overload that implies an increase in cardiac output. Our results show a direct influence of protein intake on cardiac function parameters, suggesting a possible modulation towards an increase in IGF-1 and organ development resulting from the early protein intake. These changes could suggest an early programming effect, with changes in function resulting in subsequent structural changes. However, since the effect of the type of formula on cardiac function parameters remains significant even following adjustment for IGF-1 (total as well as free), our results are unable to distinguish whether an independent IGF-1 axis mechanism would still play a role.

Protein induced metabolic consequences on the cardiovascular system

Nutrient intakes during rapid-growth phases are able to affect body structure and function. The effect of protein supply during gestation and early infancy is one of the main nutritional factors that could modulate later risk of obesity and hypertension as well as kidney size and function (4,35). Our results suggest that this mechanism is also able to influence the cardiac system through an indirect mechanism via overall growth of the body, accompanied by an increased metabolic expenditure (i.e. energy expenditure) and a consequent increased cardiac workload. Possibly, cardiac workload or whatever involves increased O_2 requirements in children with greater body surface, can determine the long-term increase in cardiac mass, as evidenced in obese individuals

(36). However, we are unable to explore this hypothesis in the 2-year-olds because of the short time-course of the study encompassing a short developmental period and the low prevalence of obese infants observed at this age. We would not expect this slight change in cardiac mass to exert health effects on healthy children. However, long-term effects if overweight persists would require further investigation. Consequently, if health consequences could be predicted by the formula diets administered to the infants, then it would be important to revise infant formula regulations. Figure 5 presents a rationale to explain our findings, particularly the main steps involved in the early phase period. The increase in early protein intake could induce an increase in IGF-1 that produced an increase in body mass with the corresponding raise in energy expenditure. A posterior adrenergic response in the sympathetic system could mediate an increase in the cardiac output, observed as increases in cardiac function parameters. These mechanisms have potential long term effects. However, we are unable to extrapolate our findings to later-stage developments.

A potential limitation of the present study is the exclusion of blood pressure measurements from 32 infants (23% of the participants) due to insufficient or unreliable measurements/readings. However, there were no significant differences in the excluded infants' with respect to feeding group distributions. A further limitation is that the IGF-1 axis determinations were performed in a single blood sample. This was unavoidable since repeated blood sampling in infants is difficult to achieve. Another limitation is sample size. The study sample did not provide sufficient statistical power to detect slight differences in cardiac mass between the feeding groups. Despite these limitations, the study provides new data obtained in a group of healthy infants that had been randomized at birth to receive infant formula diets. Confidence in the measurements was assured by high-standard anthropometric measurement protocols and

echocardiographic assessment by a single expert clinical cardiologist who was blinded with respect to the dietary provenance of the infants.

In summary our study demonstrated that a moderate increase in protein intake during the first year of life is associated not only with higher BMI but also higher cardiac function parameters at 2 years of age. However, we did not find changes in cardiac mass at this early stage of the child's development. The IGF-1 axis may partly mediate these effects by an indirect pathway that induces changes in the body size as well as on energy expenditure. Further investigation is warranted to elucidate the clinical applications of this nutritional programming effect in infancy.

METHODS

Study design and population sample

The study was performed in the Spanish subsample of infants from the EU Childhood Obesity Program (EU CHOP) trial. This study is an ongoing European collaborative randomized controlled trial in five European countries (Belgium, Germany, Italy, Poland and Spain) investigating the long-term consequences of different protein intakes during the first year of life (6). The double-blinded randomized, clinical trial [NTC00338689] comparing two groups of infants fed, during the first year of life, either higher (HP) or lower (LP) protein content formulas; both within the recommended range for desired growth and development. The primary aim of the clinical trial was to evaluate the influence of this variation in protein intake on early growth and subsequent obesity. A non-randomized group of infants exclusively breast-fed during the first 3 months of life (BF), was followed-up as a reference group.

Dietary intake and growth parameters were assessed periodically during the first two years of life (6,37). A blood sample was taken at 6 months of age. Echocardiography

and blood pressure measurements were performed at the age of 2 years.

Infant-formulas had a percentage of the energy content provided by proteins of 7.1% *vs.* 11.7% (1.25 g/100 ml *vs.* 2.05 g/100 ml for LP and HP, respectively), and follow-on formulas provided 8.8% *vs.* 17.6% of energy content (1.6 g/100 ml *vs.* 3.2 g/100 ml for LP and HP, respectively). Equal energy content in both study formulas was achieved by increasing the fat content in the LP formula. Carbohydrates and other nutrients were not significantly different. Further details on the study formulas and protocols have been published elsewhere (6). The composition of all study formulas complied with the 1991 European Union Directive on Infant and Follow-on Formulae (38) in place at the time of the study. The protein contents represented approximately the lowest and highest levels of the range recommended in this Directive.

Assessments

Anthropometry

Weight was measured using a Seca 702 scale with a precision ±0.05 Kg (Seca, Hamburg, Germany). Recumbent length was measured at birth using Seca 232 (Seca, Hamburg, Germany) and a PED LB 35–107 X (Ellard Instrumentation, Monreo, WA); both with a precision ±1 mm at the age of 24 months. Body mass index ($BMI\ [kg/m^2] = \frac{weight}{length[m]^2}$) and total body surface ($BSA\ [m^2] = \sqrt{\frac{weight\ [kg]\ x\ length\ [cm]}{3600}}$) were calculated (39). Weight-gain per month and length-gain per month from birth to 2 years were calculated. All anthropometric variables were transformed into z-scores, according to the World Health Organization Standards (WHO) (40) using the WHO Anthro for personal computers software (World Health Organization, Geneva, Switzerland).

Blood analyses

Serum parameters analyzed at 6 months of age were: total IGF-1 (ng/mL), free IGF-1 (ng/mL), IGFBP2 (ng/mL) and IGFBP3 (ng/mL). Serum samples were frozen at -70ºC until analyzed in a centralized laboratory in Warsaw (Children's Memorial Health Institute, Warsaw, Poland) (41). The IGF-1 axis analyses were performed by radioimmunoassay using commercial kits according to the manufacturer's instructions (Diagnostic Systems Laboratories, Inc., Webster, TX).

Cardiac assessment

Transthoracic Doppler echocardiography was performed by a single pediatric cardiologist (RC) to assess cardiac mass and function. The device used was VIVID 4 with a 5 MHz transductor allowing bi-dimensional M mode evaluation (General Electrics, New York, NY). The following parameters were measured in triplicate: interventricular septum thickness in systole, interventricular septum thickness in diastole (dIVS), left ventricular posterior wall in systole, left ventricular posterior wall in diastole (dLVPW), left ventricular diastolic diameter (dLVD), left ventricular systolic diameter. Calculated variables were: left ventricular mass (LVM; g), indexed left ventricular mass (LVMI; g/m$^{2.7}$), ejection fraction (EF; %) and shortening fraction (SF; %). LVM was calculated according to the recommendations of the American Society of Echocardiography as modified by

Devereux: $LVM = \{1.04[(dIVS + dLVD + dLVPW)^3 - dLVD^3]\} + 0.6$. LVMI was calculated as recommended by de Simone (25,42) as: LVMI = LVM/length$^{2.7}$.

Cardiac function was evaluated using M-Mode echocardiographic examination to determine left ventricular systolic function. Ejection fraction (EF) and shortening fraction (SF) were calculated digitally using the Teicholz method (43).

All echocardiographic measurements were performed according to the recommendations of the American Society of Echocardiography (43).

At the 2 year visit, blood pressure measurements were performed in triplicate using the oscillometric monitor Dinamap Pro 300 (precision: ±8 mmHg). The mean value was used in the statistical analyses. The appropriate cuff was applied with the subject in sitting position on the mother's lap. Not being able to obtain 3 valid blood pressure measurements was a criterion to remove this variable from the statistical analyses.

Total energy expenditure assessment

Total energy expenditure (TEE) as kcal/day was calculated using the Schofield equations (44) for basal metabolic rate

$BMR = [0.167\ x\ weight\ [kg] + 1517.4\ x\ length\ [m] - 617.6]$ or

$BMR = [16.25\ x\ weight\ [Kg] + 1023.2\ x\ length\ [m] - 413.5]$ for boys and girls, respectively. The TEE was then calculated using the physical activity levels as described by Butte et al (45) at 2 years as:

TEE (kcal/day) = BMR [kcal] x 1.4.

Data analysis

Data management and statistical analyses were carried out with the SPSS Statistics software package version 20.0 (IBM corp., Armonk, NY). Descriptive results are presented as frequencies for categorical variables and as mean (±SD) for continuous variables. Frequency distributions between feeding types were compared with the χ^2 test. Anthropometry, blood variables as well as cardiac mass and function between dietary formulas were compared with Student's *t*-test or Mann-Whitney U test (for normally and non normally distributed variables, respectively) or by ANOVA analysis

and Bonferroni *post hoc* correction for multiple testing when breastfed infants were also included in the analyses. Linear associations between cardiac variables and the other continuous variables were analyzed by Pearson or Spearman correlation coefficients, as appropriate. Linear regression models were applied to evaluate the effect of feeding type on cardiac mass and function. Adjustment was performed using anthropometric variables. Also, linear regression models were applied to determine the effect of daily protein intake during first months of life on cardiac variables, and on body weight or BMI. Statistical significance was accepted at the level of $p<0.05$.

Ethical considerations

The local Ethical Committees approved the study. All study procedures conformed to the Helsinki II declaration. Written informed parental consent was obtained for each infant. Data were codified to ensure anonymity.

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Table 1. Correlation coefficients between anthropometric variables and cardiac parameters

Anthropometric variable	**EF (%)**	**SF (%)**	**LVM (g)**
Weight for age at 24 months (z score)	0.305†	0.323†	0.354†
Length for age at 24 months (z score)	0.221**	0.241*	0.364†
BMI for age at 24 months (z score)	0.231**	0.238**	0.174*
Weight gain per month (kg/month)	0.346†	0.363†	0.402†
Length gain per month (cm/month)	0.259**	0.277**	0.361†

†: $p<0.001$, **: $p<0.01$, *: $p<0.05$

Figure legends

Figure 1. Flow chart of study participants. Randomization, follow up until 2 years of age, and cardiac assessment

Figure 2. Cardiac function parameters (EF in Panel A and SF in Panel B) segregated by feeding type. *:p<0.05 *vs.* LP

Figure 3. Effect of increase 1g/day in protein intake on: a) EF and SF; b) Weight and BMI. *: p<0.05, **: p<0.01, †: p<0.001

Figure 4. IGF-1 axis parameters segregated by feeding type. A: Total IGF-1, B: Free IGF-1, C: IGFBP2 and D: IGFBP3. *: p<0.05, **: p<0.01, †: p< 0.001 *vs.* BF; ‡: p< 0.05, §: p< 0.001 *vs.* LP

Figure 5. Possible protein-induced metabolic "programming pathways" influencing the cardiovascular system structure and function

Figure 1

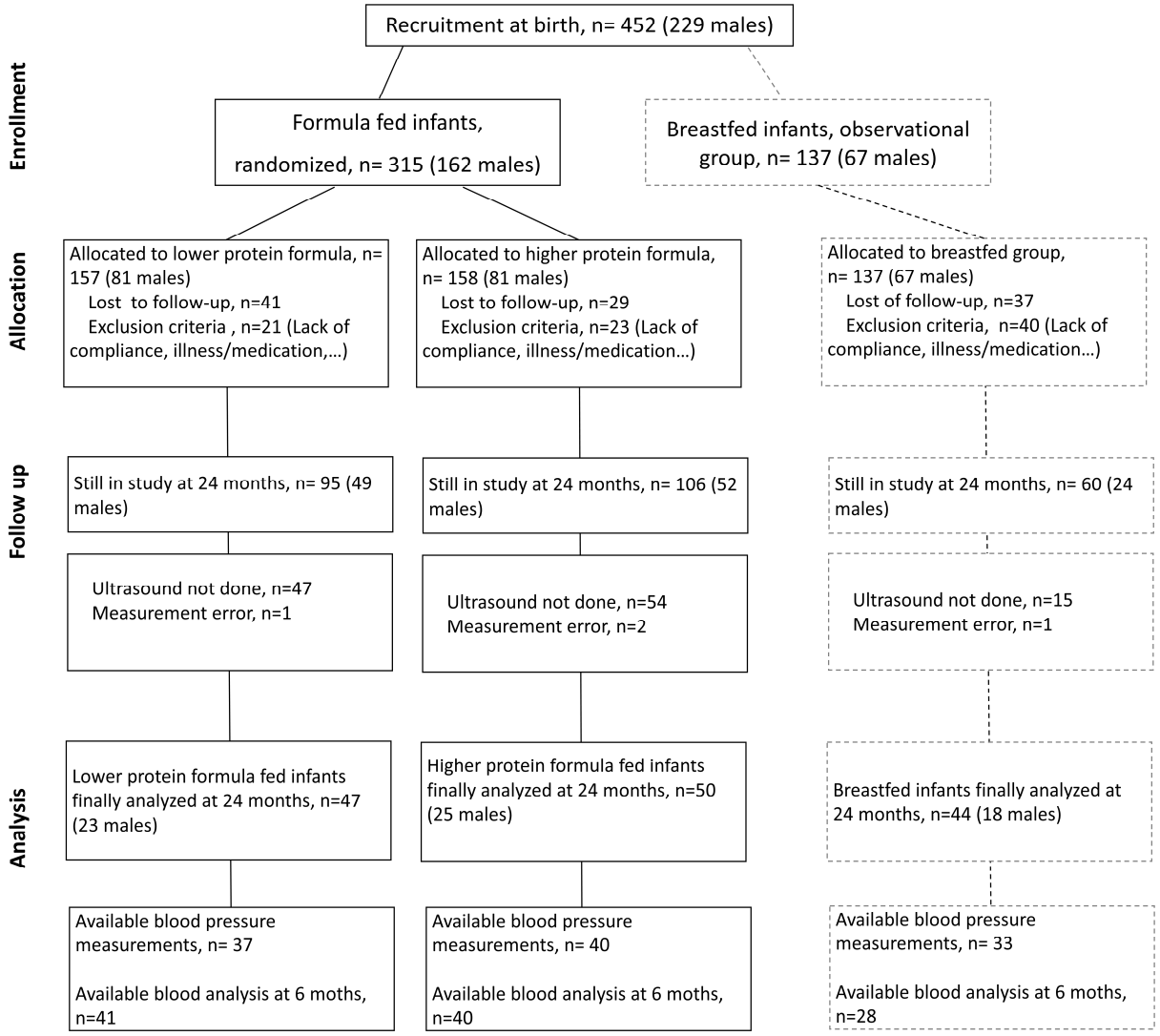

Figure 2

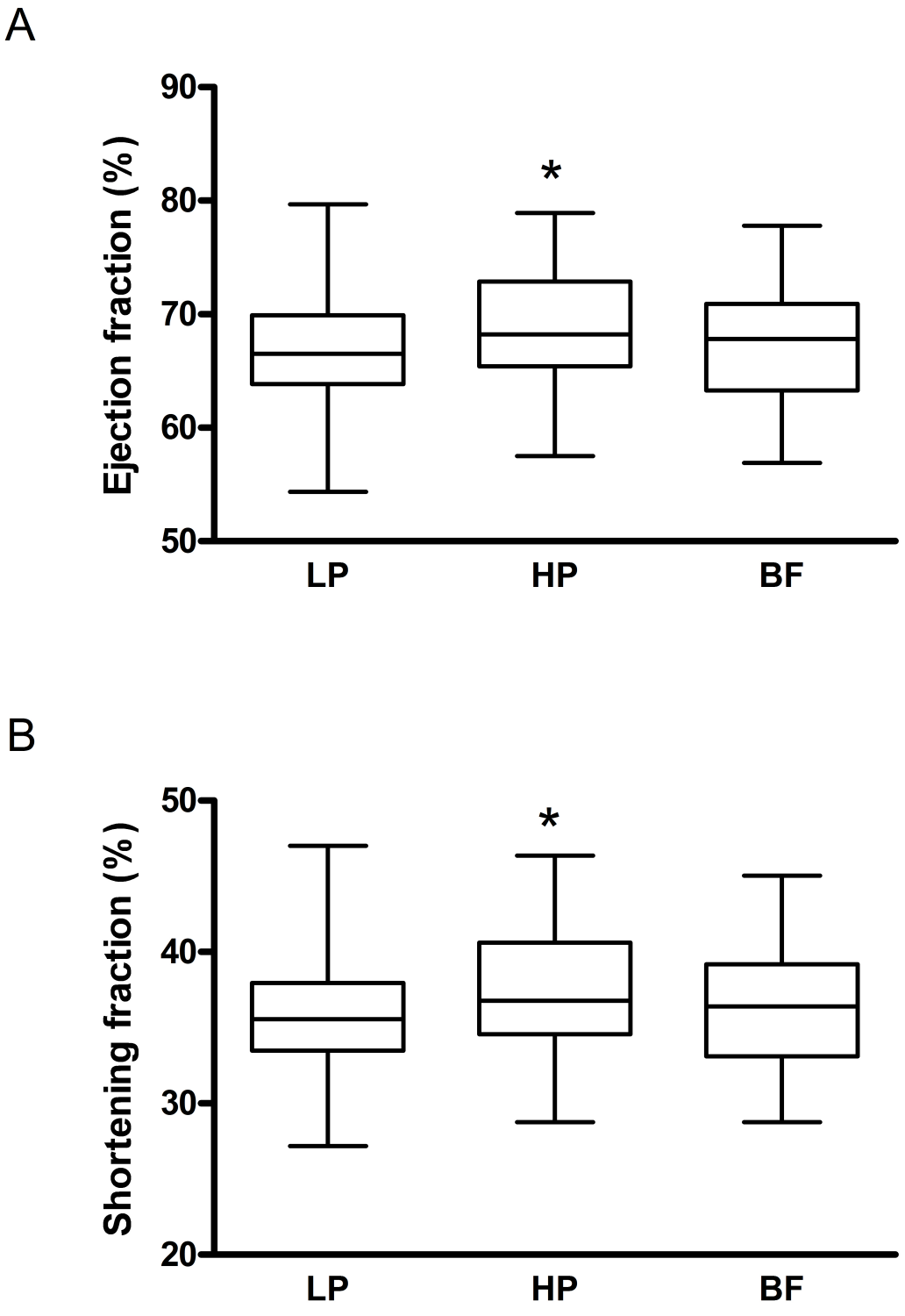

Figure 3

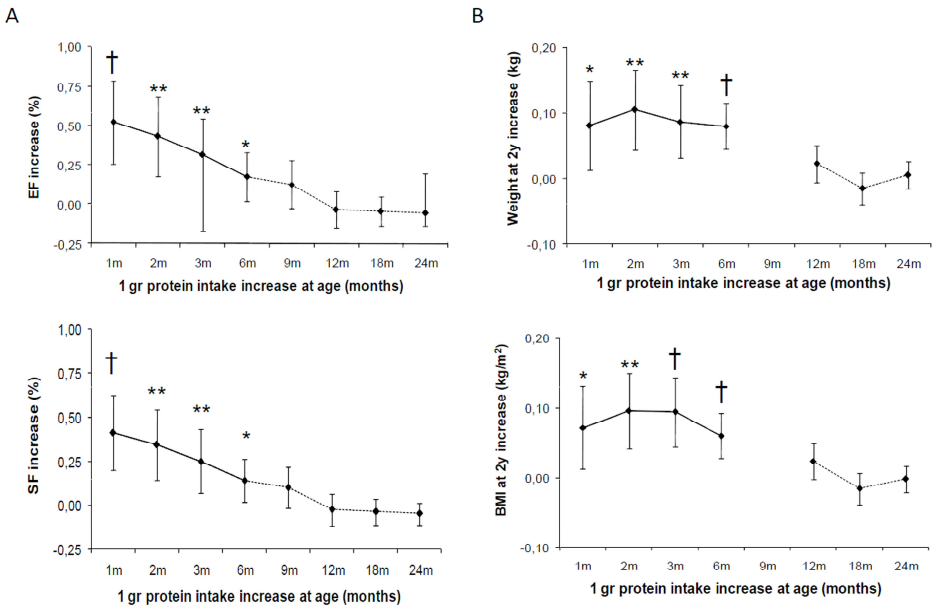

Figure 4

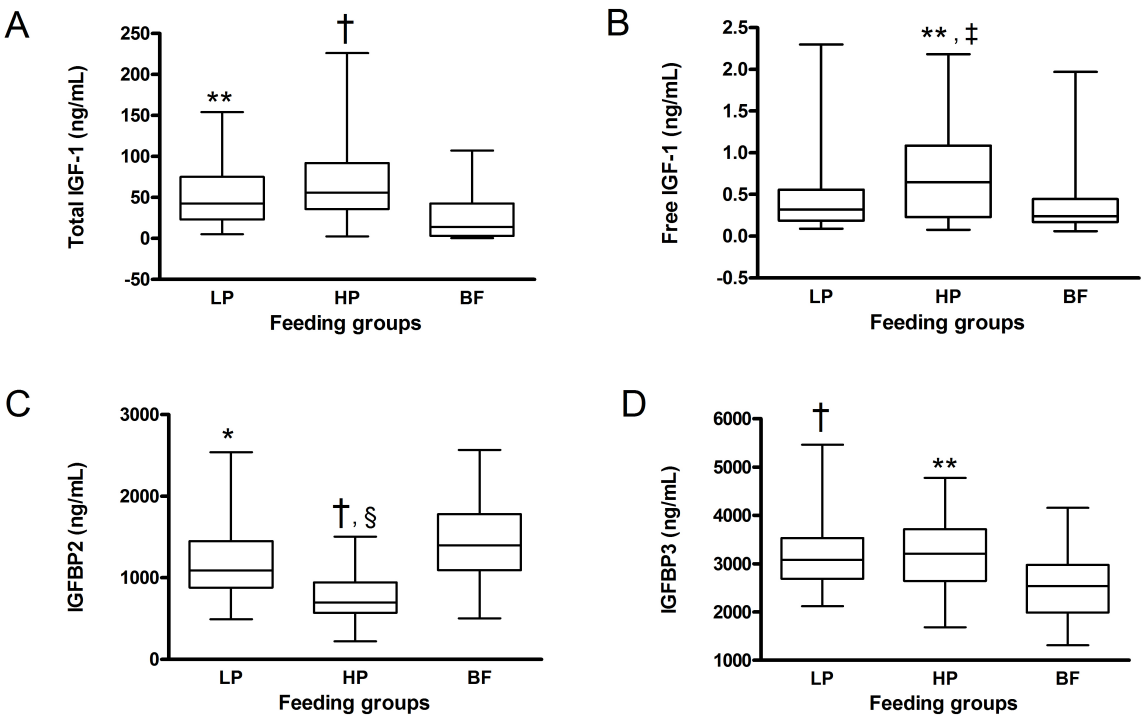

Figure 5