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OBESITY/INSULIN RESISTANCE, TYPE 2 DIABETES

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# Insulin Resistance during normal child growth and development is associated with a distinct blood metabolic phenotype (Earlybird 72)

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## Abstract

**Background:** While insulin resistance (IR) is associated with specific metabolite signatures in adults, there have been few truly longitudinal studies in healthy children, either to confirm which abnormalities are present, or to determine whether they precede or result from IR. Therefore, we investigated the association of serum metabolites with IR in childhood in the Earlybird cohort.

**Methods:** The Earlybird cohort is a well-characterized cohort of healthy children with annual measurements from age 5 to 16 years. For the first time, longitudinal association analyses between individual serum metabolites and homeostatic model assessment (HOMA) of insulin resistance (HOMA-IR) have been performed taking into account the effects of age, growth, puberty, adiposity, and physical activity.

**Results:** IR was higher in girls than in boys and was associated with increasing body mass index (BMI). In longitudinal analysis IR was associated with reduced concentrations of branched-chain amino acids (BCAA), 2-ketobutyrate, citrate and 3-hydroxybutyrate, and higher concentrations of lactate and alanine. These findings demonstrate the widespread biochemical consequences of IR for intermediary metabolism, ketogenesis, and pyruvate oxidation during normal child growth and development.

**Conclusions:** Longitudinal analysis can differentiate metabolite signatures that precede or follow the development of greater levels of IR. In healthy normal weight children, higher levels of IR are associated with reduced levels of BCAA, ketogenesis, and fuel oxidation. In contrast, elevated lactate concentrations preceded the rise in IR. These changes reveal the metabolite signature of insulin action during normal growth, and they contrast with previous findings in obese children and adults that represent the consequences of IR and obesity.

KEYWORDS
amino acids, biochemistry, insulin resistance, longitudinal, phenotyping

## 1 | INTRODUCTION

Epidemic obesity has resulted in a rapid increase of type 2 diabetes (T2D) in children.[1-3] However, the pathways linking childhood obesity to T2D remain poorly characterized, and prediction and prevention of T2D are unfulfilled aspirations. Although insulin resistance (IR) is important in the pathophysiology of T2D, the causes of IR in children are complex, because they include both normal physiological changes and weight gain. Therefore, there is a need to understand the interplay of normal physiological factors and pathophysiological parameters that contribute to IR in children. Metabolic phenotyping is now a well-established approach to characterizing complex metabolic processes, and investigating the molecular origins of IR in children.[4]

Metabolic phenotypes, or metabotypes, reflect systemic influences on molecular regulatory processes, including dietary and physiological factors.[5] In children, longitudinal analysis of metabotypes has the potential to unravel the complex biochemistry of puberty, molecular disturbances associated with IR, and to identify individuals at risk of T2D.[6,7] In previous cross-sectional studies of adults, branched chain and aromatic amino acid metabotypes were consistently and positively associated with IR, prediabetes and T2D, independently of adiposity.[8] Furthermore, a meta-analysis of eight prospective studies showed that each study-specific SD difference in isoleucine, leucine, and tyrosine was associated with a 36% higher risk of T2D. Similarly, valine and phenylalanine were associated with 35% and 26% increased risk of T2D, while glycine and glutamine were inversely associated with T2D risk. These associations have led to the suggestion that derangement of branched-chain amino acid (BCAA) catabolism may be an important mechanism in the pathway to IR.

In order to test such hypotheses, longitudinal studies in children are necessary. However, there have been very few truly longitudinal studies in children. A review of 10 studies in children found that BCAA, aromatic amino acids (AAA), and acylcarnitines were associated with IR, and BCAA and tyrosine were associated with future metabolic risk.[9] However, most of these studies were cross-sectional in predominantly obese children. In contrast, there has been a lack of formal longitudinal studies of normal weight children. Therefore, it remains uncertain whether the metabolic changes associated with IR in reported studies represent cause or effect.

The EarlyBird study is a longitudinal cohort study of healthy children, with annual clinical, anthropometric, and physiological assessment from age 5 to 16 years. This study provides an ideal opportunity for a detailed longitudinal metabonomic analysis investigating how metabotypes relate to IR during childhood and adolescence. To our knowledge, this is the first time the association between individual metabolites and IR (homeostaticmodel assessment [HOMA] of insulin resistance [HOMA-IR]) in childhood has been investigated in a detailed longitudinal study, taking into account the effects of critical covariates such as age, growth, puberty, adiposity, and physical activity.

## 2 | METHODS

### 2.1 | Study population

We conducted the study in accordance with the ethical guidelines of the Declaration of Helsinki II; ethics approval was granted by the Plymouth Local Research Ethics Committee (1999), and parents gave written consent and children verbal assent. The EarlyBird Diabetes Study incorporates a 1995/1996 birth cohort recruited in 2000/2001 when the children were 5 years old (307 children, 170 boys).[10] The collection of data from the EarlyBird cohort is composed of several clinical and anthropometric variables measured on an annual basis from the age of 5 to the age of 16. Details on the measurement methods are reported hereafter and in Supplementary Materials.

A first study was undertaken on 40 participants from age 5-14 years (Study 1), and this was then extended to a full analysis of 150 participants from age 5 to 16 (Study 2). In Study 1, 40 subjects were chosen on the basis of having a complete set of samples available for analysis at each time-point between 5 and 14 years (20 boys), having been stratified by IR (1st and 4th quartile) at 5 and 14 years. This study was designed as a pilot study to explore whether IR was associated with specific metabotypes, HOMA-IR being used as an indicator of deterioration of glucose control. The Study 2 aims at replicating the observations in higher number of children, and extending the analysis till the age of 16 years when samples became available. In Study 2, subjects were purposively selected to include all children who had shown impaired fasting glucose (at one or more time-points during the course of the childhood as reported previously[11]), and gender-matched normoglycemic children, resulting in a total of 150 participants. Out of the 55 children who had shown impaired fasting glucose in this subset, seven had a first degree relative with T2D or T1D. Impaired fasting glycaemia has been selected as an objective criterion to select children with an additional risk for future diabetes. This resulted in the selection of 105 boys and 45 girls. In Study 2, children had completed their visits at year 15 and 16, providing a complete view on IR and glucose variations in adolescence. Twenty children were common among the two studies.

### 2.2 | Clinical and anthropometric assessments

IR was determined each year from fasting glucose (Cobas Integra 700 analyzer; Roche Diagnostics, Basel, Switzerland) and insulin (DPC IMMULITE, Siemens, California) (cross-reactivity with proinsulin, 1%) using the homeostasis model assessment program,[12,13] which has been validated in children.[14] BMI was derived from direct measurement of height (Leicester Height Measure; Child Growth Foundation, London, UK) and weight (Tanita Solar 1632 electronic scales), performed in blind duplicate and averaged. BMI z-scores were calculated from the British 1990 standards.[15] Moderate to vigorous physical activity (MVPA) was measured annually from 5 years by accelerometry (Acti-Graph, Actigraph, model7164, Computer Science and Applications Inc, Shalimar, Florida).[16] Children were asked to wear the accelerometers for seven consecutive days at each annual follow-up visit, and only recordings that captured at least 4 days were used. Pubertal timing was evaluated by means of age

**TABLE 1** Characteristics of the cohort at 5y and 14y by gender

| | | Boys | Girls |
|---|---|---|---|
| Age (years) | 5y | 5.1 (4.8-5.3) | 4.8 (4.7-5.0) |
| | 14y | 13.9 (13.6-14.1) | 13.8 (13.7-14.1) |
| BMI z-scores | 5y | −0.04 (−0.50-0.72) | 0.40 (0.04-0.80) |
| | 14y | 0.43 (−0.13-1.29) | 0.78 (−0.05-1.48) |
| Moderate-vigorous physical activity (minutes/day) | 5y | 46.1 (34.1-67.1) | 55.1 (44.9-62.5) |
| | 14y | 52.4 (29.7-77.0) | 42.7 (29.3-51.4) |
| Age at peak height velocity (years) | | 13.4 (12.9-13.8) | 11.9 (11.1-12.5) |
| IR (HOMA2-IR) | 5y | 0.47 (0.37-0.84) | 0.85 (0.34-1.02) |
| | 14y | 1.25 (0.56-1.63) | 0.98 (0.78-2.23) |

*Note*: Data are median (interquartile range).
Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance.

at peak height velocity (APHV), determined as the tangential velocity at the middle time point of three consecutive height measurements taken 6 months apart. Data are reported in Tables 1 and 2. Peripheral blood was collected annually after an overnight fast, blood serum were stored at −80°C.

## 2.3 | Metabonomics

Serum samples collected from each child at every age between 5 and 16 years old were subjected to metabonomics. For technical feasibility and to ensure optimal data reproducibility for cohort analysis, a threshold of 1800 blood serum samples (eg, 150 different subjects) was determined. Metabolic profiling was carried out by means of Proton nuclear magnetic resonance spectroscopy ($^1$H NMR) spectroscopy, as reported previously.[6] Briefly, 400 μL of blood serum were mixed with 200 μL of deuterated phosphate buffer solution 0.6 M $KH_2PO_4$. $^1$H NMR metabolic profiles of serum samples were acquired with a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm cryoprobe at 310 K (Bruker Biospin, Rheinstetten, Germany) and processed using TOPSPIN (version 2.1; Bruker Biospin) software package. Based on an internal database of reference compounds, representative signals of metabolites were integrated. The signals are expressed in an arbitrary unit corresponding to a peak area normalized to total metabolic profiles. $^1$H NMR spectroscopy being a quantitative methods, metabolite peak area are proportional to metabolite concentrations, and thus their changes are representative of absolute change in metabolite concentration in the serum. This metabonomics approach covers major metabolic pathways, including lipoproteins, amino acids, carboxylic acids, and central energy metabolism in a highly reproducible manner across more than 1700 serum samples. In particular, $^1$H-NMR spectrum of human blood serum enables the monitoring of signals related to lipoprotein bound fatty acyl groups found in triglycerides, phospholipids, and cholesteryl esters, together with peaks from the glyceryl moiety of triglycerides and the choline head group of phosphatidylcholine. In relation to technological developments, the analysis of Study 2 resulted in more sensitive $^1$H NMR data and detection of a slightly greater number of low abundant metabolites.

**TABLE 2** Characteristics of the main cohort at 5y, 14y, and 16y by gender

| | | Boys | Girls |
|---|---|---|---|
| Age (years) | 5y | 4.8 (4.7–5.0) | 4.9 (4.8-5.1) |
| | 14y | 13.8 (13.6-14.0) | 13.9 (13.8-14.0) |
| | 16y | 15.8 (15.6-16.0) | 15.9 (15.8-16.1) |
| BMI z-scores | 5y | 0.09 (−0.48-0.80) | 0.36 (−0.49-1.16) |
| | 14y | 0.32 (−0.64-0.98) | 0.85 (0.21-1.60) |
| | 16y | 0.44 (−0.31-1.32) | 0.70 (0.03-1.60) |
| Moderate-vigorous physical activity (minutes/day) | 5y | 53.1 (41.6-64.1) | 40.1 (31.9-51.6) |
| | 14y | 44.4 (30.7-63.14) | 35.3 (18.4-50.4) |
| | 16y | 43.9 (22.5-58.6) | 27.0 (16.8-40.9) |
| Age at peak height velocity (years) | | 13.0 (12.8-13.4) | 11.6 (10.8-12.3) |
| IR (HOMA2-IR) | 5y | 0.49 (0.22-0.74) | 0.76 (0.60-1.00) |
| | 14y | 1.00 (0.79-1.47) | 1.47 (1.00-1.70) |
| | 16y | 0.70 (0.23-1.16) | 0.84 (0.23-1.18) |

*Note*: Data are median (interquartile range).
Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin resistance.

## 2.4 | Statistical analysis

The distribution of the outcome variable, IR, was skewed and so log-transformed (Log IR) while each metabolite was transformed to a z-score (ie, standardized with mean of 0 and SD of 1) for analysis. For both Study 1 and Study 2, mixed effects modeling was used to assess the association between individual metabolites and IR (HOMA-IR), taking into account age, BMI z-scores, and physical activity. Controlling for maturational and growth status is crucial in life course studies, and APHV is a key measure of maturity that was also taken into account. Random intercepts were included as well as age (categorized to allow for non-linear change in IR over time), gender, BMI z-score, APHV, MVPA (number of minutes spent in moderate-vigorous physical activity), and individual metabolites (in separate models) as fixed effects. Unadjusted and Bonferroni adjusted *P*-values are reported in Tables 3 and 4. Because insulin was log transformed the exponentiated coefficients represent the expected percent change in IR associated with a unit (SD) change in metabolite. Because one objective was to find replicated associations between studies, the analysis conducted in Study 2 has been focused only on serum metabolites previously detected in Study 1 serum samples. Only succinate was not consistently detected in all samples from Study 2 and therefore analysis not repeated. Results obtained on metabolites only detected in Study 2 are provided in Supplementary Table 1.

**TABLE 3** Estimates and *P*-values from mixed effects models examining the association between metabolites and IR in Study 1 (n = 40)

| Variable ID | Metabolites | $^{1}$H NMR chemical shift of the integrated signal (ppm) | Coef | SE | *P*-value (unadjusted) | Bonferroni-adjusted *P*-value |
|---|---|---|---|---|---|---|
| 1 | Lipid (mainly HDL, fatty acid CH3 moieties) | 0.83 | −0.052 | 0.03 | .08266 | 1.00 |
| 2 | Lipid (mainly LDL, fatty acid CH3 moieties) | 0.87 | 0.056 | 0.028 | .04781 | 1.00 |
| 3 | Leucine | 0.96 | −0.103 | 0.027 | .00015 | .01 |
| 4 | Isoleucine | 1.01 | −0.045 | 0.026 | .08713 | 1.00 |
| 5 | Valine | 1.05 | −0.107 | 0.026 | 4.6E-05 | .003 |
| 6 | 2-Ketobutyrate | 1.07 | −0.089 | 0.037 | .01695 | 1.00 |
| 7 | 3-D-hydroxybutyrate | 1.18 | −0.106 | 0.027 | .00013 | .01 |
| 8 | Lipid (mainly HDL, fatty acid CH3 moieties) | 1.23 | −0.012 | 0.03 | .68734 | 1.00 |
| 9 | Lipid (mainly LDL, fatty acid [CH2]n moieties) | 1.27 | 0.087 | 0.028 | .00227 | .13 |
| 10 | Lactate | 1.33 | 0.067 | 0.025 | .00719 | .42 |
| 11 | Alanine | 1.48 | 0.085 | 0.024 | .00044 | .03 |
| 12 | Lipid (mainly VLDL, fatty acid [CH2] moieties) | 1.5 | 0.043 | 0.023 | .06153 | 1.00 |
| 13 | Arginine | 1.71 | −0.077 | 0.029 | .00751 | .44 |
| 14 | Lysine | 1.76 | −0.024 | 0.023 | .28897 | 1.00 |
| 15 | Acetate | 1.91 | −0.01 | 0.025 | .69837 | 1.00 |
| 16 | N-acetyl proteins | 2.03 | 0.024 | 0.028 | .39399 | 1.00 |
| 17 | Glutamate | 2.12 | −0.034 | 0.025 | .17895 | 1.00 |
| 18 | 3-D-hydroxybutyrate | 2.3 | −0.084 | 0.024 | .0004 | .02 |
| 19 | Glutamate | 2.35 | 0.027 | 0.026 | .31137 | 1.00 |
| 20 | Glutamine | 2.45 | −0.04 | 0.026 | .12765 | 1.00 |
| 21 | Citrate | 2.66 | −0.132 | 0.028 | 3E-06 | .0002 |
| 22 | Asparagine | 2.85 | −0.028 | 0.025 | .26311 | 1.00 |
| 23 | Trimethylamine | 2.87 | −0.06 | 0.024 | .01141 | .67 |
| 24 | Dimethylglycine | 2.93 | −0.046 | 0.026 | .08207 | 1.00 |
| 25 | Lysine | 3 | −0.06 | 0.027 | .02559 | 1.00 |
| 26 | Creatine | 3.02 | −0.095 | 0.029 | .0011 | .06 |
| 27 | Citrulline | 3.14 | −0.081 | 0.032 | .01143 | .67 |
| 28 | Phospholipids | 3.21 | −0.131 | 0.031 | 3.8E-05 | .002 |
| 29 | Glucose | 3.25 | −0.004 | 0.026 | .88223 | 1.00 |
| 30 | Trimethylamine-N-Oxide | 3.25 | −0.028 | 0.029 | .32906 | 1.00 |
| 31 | Taurine | 3.29 | −0.008 | 0.029 | .78819 | 1.00 |
| 32 | Proline | 3.34 | 0.03 | 0.027 | .26484 | 1.00 |
| 33 | Glycine | 3.57 | 0.007 | 0.03 | .81812 | 1.00 |
| 34 | Creatine | 3.93 | −0.058 | 0.033 | .08599 | 1.00 |
| 35 | Serine | 3.96 | −0.002 | 0.03 | .93441 | 1.00 |
| 36 | Threonine | 4.26 | 0.013 | 0.023 | .56905 | 1.00 |
| 37 | Glucose | 5.23 | −0.003 | 0.025 | .89908 | 1.00 |
| 38 | Histidine | 7.06 | −0.049 | 0.026 | .0588 | 1.00 |
| 39 | Tyrosine | 7.2 | −0.005 | 0.026 | .8485 | 1.00 |
| 40 | Histidine | 7.83 | −0.057 | 0.027 | .03601 | 1.00 |
| 41 | Formate | 8.45 | −0.003 | 0.025 | .91772 | 1.00 |
| 42 | Succinate | 2.39 | −0.042 | 0.025 | .09536 | 1.00 |

*Note*: Coef: Coefficient indicating the directions of the associations between the metabolite and Log IR overtime. Because insulin was log transformed the exponentiated coefficients represent the expected percent change in insulin resistance associated with a unit (SD) change in metabolite. SE: standard error for the coefficient.

Abbreviation: IR, insulin resistance; LDL, low density lipoproteinsl; VLDL, very low density lipoproteins.

**TABLE 4** Estimates and *P*-values from mixed effects models examining the association between metabolites and IR in Study 2 (n = 150)

| Variable ID | Metabolites | $^{1}$H NMR chemical shift of the integrated signal (ppm) | Coef | SE | *P*-value (unadjusted) | Bonferroni adjusted *P*-value |
|---|---|---|---|---|---|---|
| 1 | Lipid (mainly HDL, fatty acid CH3 moieties) | 0.83 | −0.059 | 0.024 | .012 | .96 |
| 2 | Lipid (mainly LDL, fatty acid CH3 moieties) | 0.87 | 0.108 | 0.023 | <.0001 | .0006 |
| 3 | Leucine | 0.96 | −0.121 | 0.019 | <.0001 | <.0001 |
| 4 | Isoleucine | 1.01 | −0.05 | 0.021 | .0178 | 1.00 |
| 5 | Valine | 1.05 | −0.114 | 0.02 | <.0001 | <.0001 |
| 6 | 2-Ketobutyrate | 1.07 | −0.071 | 0.019 | .0003 | .024 |
| 7 | 3-D-hydroxybutyrate | 1.18 | −0.092 | 0.018 | <.0001 | <.0001 |
| 8 | Lipid (mainly HDL, fatty acid CH3 moieties) | 1.23 | 0.052 | 0.021 | .0156 | 1.00 |
| 9 | Lipid (mainly LDL, fatty acid [CH2]n moieties) | 1.27 | 0.133 | 0.023 | <.0001 | .00093 |
| 10 | Lactate | 1.33 | 0.101 | 0.019 | <.0001 | <.0001 |
| 11 | Alanine | 1.48 | 0.156 | 0.019 | <.0001 | <.0001 |
| 12 | Lipid (mainly VLDL, fatty acid [CH2] moieties) | 1.5 | −0.12 | 0.022 | <.0001 | <.0001 |
| 13 | Arginine | 1.71 | −0.116 | 0.021 | <.0001 | <.0001 |
| 14 | Lysine | 1.76 | −0.112 | 0.019 | <.0001 | <.0001 |
| 15 | Acetate | 1.91 | −0.088 | 0.028 | .0016 | .128 |
| 16 | N-acetyl proteins | 2.03 | −0.046 | 0.022 | .0398 | 1.00 |
| 17 | Glutamate | 2.12 | −0.112 | 0.021 | <.0001 | <.0001 |
| 18 | 3-D-hydroxybutyrate | 2.3 | −0.118 | 0.019 | <.0001 | <.0001 |
| 19 | Glutamate | 2.35 | 0.042 | 0.024 | .0847 | 1.00 |
| 20 | Glutamine | 2.45 | −0.118 | 0.022 | <.0001 | <.0001 |
| 21 | Citrate | 2.66 | −0.188 | 0.021 | <.0001 | <.0001 |
| 22 | Asparagine | 2.85 | −0.115 | 0.021 | <.0001 | <.0001 |
| 23 | Trimethylamine | 2.87 | −0.123 | 0.022 | <.0001 | <.0001 |
| 24 | Dimethylglycine | 2.93 | −0.118 | 0.021 | <.0001 | <.0001 |
| 25 | Lysine | 3 | −0.139 | 0.02 | <.0001 | <.0001 |
| 26 | Creatine | 3.02 | −0.142 | 0.023 | <.0001 | <.0001 |
| 27 | Citrulline | 3.14 | −0.137 | 0.022 | <.0001 | <.0001 |
| 28 | Phospholipids | 3.21 | −0.066 | 0.024 | .0067 | .536 |
| 29 | Glucose | 3.25 | −0.017 | 0.023 | .4585 | 1.00 |
| 30 | Trimethylamine-N-Oxide | 3.25 | −0.052 | 0.021 | .0156 | 1.00 |
| 31 | Taurine | 3.29 | −0.049 | 0.02 | .0146 | 1.00 |
| 32 | Proline | 3.34 | 0.032 | 0.022 | .1442 | 1.00 |
| 33 | Glycine | 3.57 | −0.08 | 0.026 | .0023 | .184 |
| 34 | Creatine | 3.93 | −0.121 | 0.025 | <.0001 | .00013 |
| 35 | Serine | 3.96 | −0.134 | 0.022 | <.0001 | <.0001 |
| 36 | Threonine | 4.26 | −0.026 | 0.017 | .1129 | 1.00 |
| 37 | Glucose | 5.23 | −0.008 | 0.022 | .7038 | 1.00 |
| 38 | Histidine | 7.06 | −0.136 | 0.021 | <.0001 | <.0001 |
| 39 | Tyrosine | 7.2 | −0.008 | 0.02 | .6885 | 1.00 |
| 40 | Histidine | 7.83 | −0.124 | 0.022 | <.0001 | <.0001 |
| 41 | Formate | 8.45 | NA | NA | NA | NA |
| 42 | Succinate | 2.39 | NA | NA | NA | NA |

*Note*: Variable corresponding to succinate could not be integrated in Study 2. Coef: Coefficient indicating the directions of the associations between the metabolite and Log IR overtime. Because insulin was log transformed the exponentiated coefficients represent the expected percent change in insulin resistance associated with a unit (SD) change in metabolite. SE: standard error for the coefficient.

Abbreviation: IR, insulin resistance; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

To assess further which IR-associated metabolite may be an early indicator of IR trajectories, we stratified the Study 2 population according to low or high IR status over the 14-16 year age range. Arbitrarily, the 91st centile for the HOMA-IR distribution was employed as a threshold to define children with high IR status, similar to the commonly used threshold for BMI z-score stratification of weight status. Here, mixed effects modeling taking into account covariates as described above, was used to assess the association between specific metabolites and IR groups. Mixed effects modeling not taking into account covariates are reported as Supplementary Materials. Modeling was carried out in R software (www.R-project.org) using the lmer function in the package lme4[17] and *P*-values calculated using the Satterthwaite approximation implemented in the lmerTest package.[18] Cross-sectional association between metabolites and IR was assessed using Pearson's correlations at each age (Supplementary Materials). Similarly, a year-on-year association of the metabolites was assessed using Pearson's correlations (Supplementary Materials).

## 3 | RESULTS

### 3.1 | Demographics

Clinical and anthropometrics characteristics of the children in Study 1 at 5 and 14 years are summarized in Table 1 and those in Study 2 at 5, 14, and 16 years in Table 2. For both genders there HOMA-IR index decreased until around 8 years (Supplementary Figures 1a and 2a), which was followed by an increase through puberty, this trend being dependent on the time of peak height velocity (age*APHV interaction $P < .001$, Supplementary Figures 1b and 2b). IR was also positively associated with BMI z-scores ($P < .001$).

### 3.2 | HOMA-IR and serum metabolite associations in childhood

In Study 1, several metabolites including amino acids, organic acids, and lipids showed a significant positive association with HOMA-IR in longitudinal models, independently of BMI z-scores, physical activity and APHV, and after multiple testing correction (Table 3). The outcomes are repeated in the analysis conducted in Study 2 (Table 4). Additional cross-sectional correlations between metabolites and HOMA-IR, and year-to-year metabolite correlations are reported in Supplementary Tables 2 and 3, for Study 2.

#### 3.2.1 | Branched-chain amino acid metabolism

In Study 1, mixed effects models showed an inverse association of valine and leucine with Log IR ($P < .001$), and a trend between isoleucine and Log IR ($P = .087$). In Study 2, the inverse association of valine and leucine with Log IR was confirmed ($P < .001$), and a weak inverse association between isoleucine and Log IR was noted ($P = .02$). Cross-sectional associations were variable according to time-point (Supplementary Table 2). Cross-sectional correlations indicated an inverse association between valine and Log IR, significant at 7, 11, and 12 years; and with leucine at 7 and 9 years only. There were statistically significant correlations between isoleucine and Log IR, first negative at 9 years, and then positive at 13 and 15 years. For leucine and valine, there was also a statistically significant gender interaction indicating that the observed association between these BCAA and Log IR was dependent upon gender. Furthermore, year-to-year correlations for valine, leucine, and isoleucine ranged from r = 0.06-0.58, 0.04-0.64, and 0.17-0.68 respectively, indicating that within-child tracking of the BCAA varied with age (Supplementary Table 3). There was little change over time in valine, leucine, and isoleucine.

Mixed effects modeling also captured the inverse association between 2-ketobutyrate and Log IR in Study 1 ($P < .01$) and Study 2 ($P < .001$). The cross-sectional correlations between 2-ketobutyrate and Log IR were statistically significant at 7, 9, and 11 years.

#### 3.2.2 | Central energy-related metabolites

In Study 1 and Study 2, mixed effects modeling described statistically significant inverse associations of citrate and 3-D-hydroxybutyrate with Log IR, and positive associations of lactate and alanine with Log IR (Tables 3 and 4). Cross-sectional correlation analyses revealed negative associations at each time point between citrate and Log IR from age 5 to 16, and between 3-D-hydroxybutyrate and Log IR from age 5 to 15 (Supplementary Table 2). In addition, Log IR was positively correlated with alanine at each time point from age 5 to 9, 11 and 12 years. Lactate was positively associated with Log IR at 7 years, 9 years, and each time point from age 11 to 16.

Furthermore, year-to-year correlations for alanine, lactate, 3-D-hydroxybutyrate and citrate ranged from r = 0.01-0.60, 0.12-0.56, 0.24-0.47, and 0.27-0.66 respectively, indicating that within-child tracking of the metabolite varied with age (Supplementary Table 3).

#### 3.2.3 | Lipid related metabolites associated with IR

In both Study 1 and Study 2, signals derived from the methyl fatty acyl groups in phospholipids containing choline showed inverse associations with Log IR, whereas signals derived from the methyl fatty acyl groups in low density lipoproteins (LDL) particles showed positive associations with Log IR. Cross-sectional associations between phospholipids and Log IR were inverse and statistically significant from age 7 to 16 years, whereas those between Log IR and fatty acyl groups in LDL particles were positive and statistically significant between 7 and 14 years. Furthermore, year-to-year correlations for phospholipids were very high ranging from r = 0.46-0.78 (Supplementary Table 3).

#### 3.2.4 | Amino acid metabolism

Mixed effects modeling identified significant inverse associations between histidine, creatine, arginine, citrulline, and lysine with Log IR in Study 1 and Study 2. Each metabolite also showed inverse cross-sectional correlations with Log IR. Correlations were significant for histidine between 9 and 13 years; creatine at 7, 9, 11, and 12 years;

arginine at 7, 8, and 12 years; citrulline between 8 and 16 years, and lysine between 7 and 16 years. Furthermore, year-to-year correlations for histidine and creatine were ranging from r = 0.19-0.70 and r = 0.25-0.71, respectively (Supplementary Table 3).

### 3.3 | Metabolites according to high IR status (91st centile) at 14-16 years

For each metabolite showing a significant longitudinal association with Log IR, we investigated whether their serum concentrations were informative for high IR status over the 14-16-year age range. Arbitrarily, the 91st centile for the HOMA-IR distribution was selected as a threshold to define high IR status. Mixed effects modeling not corrected for covariates (gender, age, BMI z-scores, physical activity, and APHV) identified a significant positive association between lactate and Log IR ($P < .001$), and fatty acyl groups in LDL ($P < .005$). Further, a significant negative association was found between Log IR and very low density lipoproteins (VLDL) particles ($P = .03$), acetate ($P = .006$), citrate ($P = .006$), asparagine ($P = .002$), trimethylamine ($P = .001$), dimethylamine ($P = .004$), lysine ($P = .005$), citrulline ($P = .007$), glycine ($P = .006$), histidine ($P = .015$), and 3-D-hydroxybutyrate ($P = .04$). Fat mass (waist circumference) was also a statistically significant variable which increased in the high Log IR group over time ($P = 2.610^{-5}$). However, when correcting for main covariates, only the association between lactate and Log IR remained significant (Supplementary Table 4). BMI z-score was an important

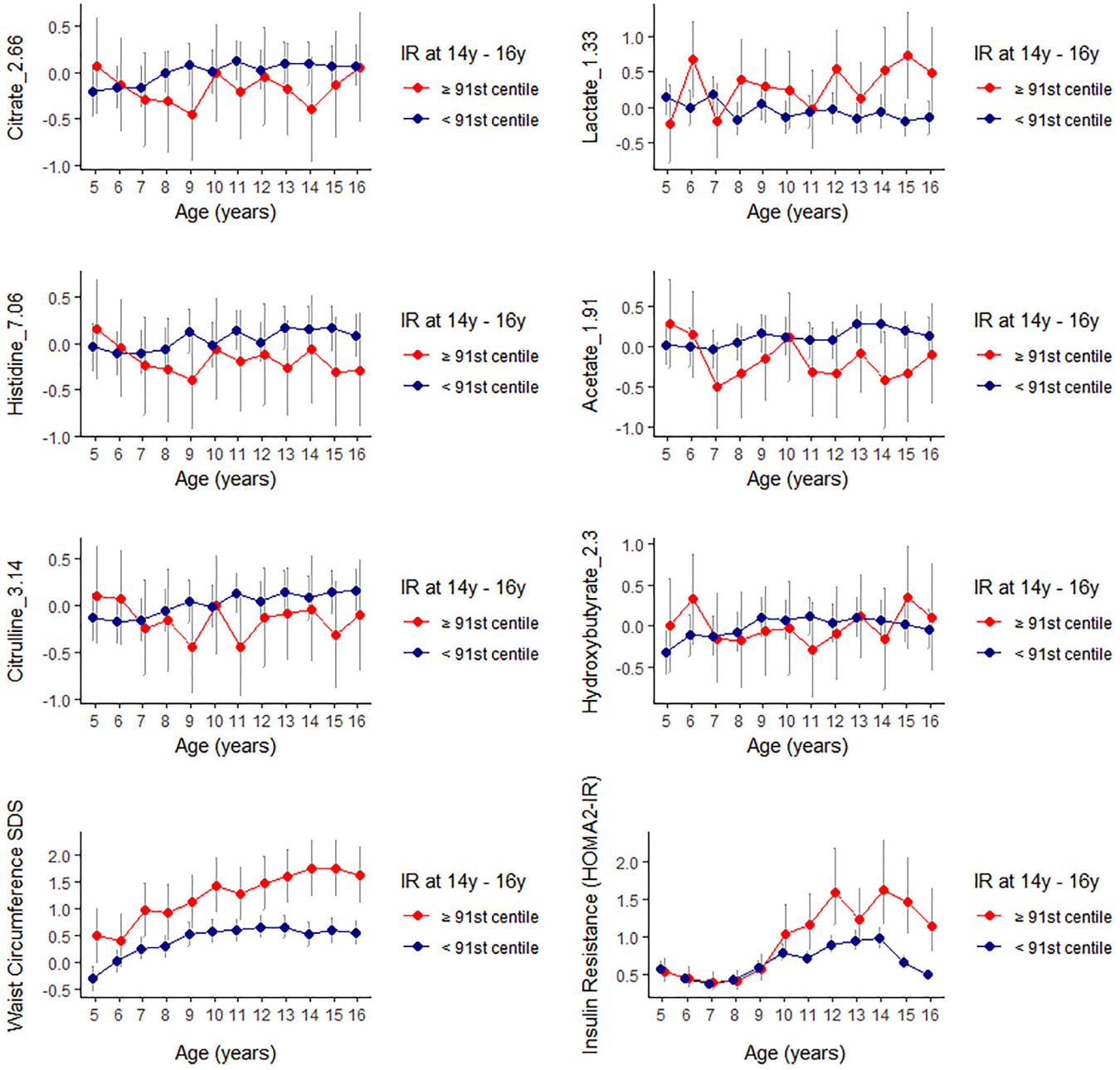


**FIGURE 1** Selected metabolites from 5y to 16y according to classification of IR between 14y and 16y (at least one occurrence of insulin resistance ≥91st centile)

covariate influencing the other observed associations. The temporal profiles of selected parameters are reported in Figure 1 and supplementary Figure 3.

## 4 | DISCUSSION

In this uniquely well-characterized cohort of healthy children, relative increases in IR are accompanied by a complex molecular signature in central energy pathways, amino acid and fatty acid metabolism. This suggests that higher levels of IR are associated with age-dependent molecular changes throughout normal growth and adolescence. The findings add to, contrast with, and significantly extend the existing literature.

Previous studies observed positive associations between BCAA concentrations and IR. In this study, Log IR was inversely associated with BCAA and 2-ketobutyrate. This difference may be because most previous studies were undertaken in adults.[19-23] In contrast, a series of relatively small studies have been reported in children,[24-34] and with limited exceptions most of these were cross-sectional. In the mainly cross-sectional studies of obese children included in the systematic review of Zhao et al, IR was found to be positively associated with BCAA.[9] Notwithstanding the limitations of these studies, the associations resemble those found in adults.[19-22,35] Therefore, our findings in mainly normal weight healthy children may differ from studies in overweight and obese children and adults. In our study, only when stratifying children according to a 91st centile of HOMA-IR at 14 to 16 years of age, BCAA levels tended to be positively associated with Log IR, yet not significantly. As pointed out by McCormack,[30] obesity may strongly influence BCAA levels by multiple mechanisms (over nutrition, protein, and lipid metabolism) associated with IR. In normal weight children, however, metabolism of BCAA may be different due to lower insulin levels and the predominant effects of normal growth and development.

Several unifying models have been proposed to explain associations between multiple metabolites and IR. Adams postulated a pivotal role for the mitochondrial enzyme Branched Chain Keto acid Dehydrogenase (BCKD) in the generation of elevated BCAA and branched chain keto acids in IR and obesity.[35] Furthermore, impairment of BCKD activity has been proposed as an explanation for elevated concentrations of aromatic and sulfated amino acids, 3-D-hydroxybutyrate and 2-ketobutyrate, all of which have also been observed in individuals with IR and obesity.[35] In our study, 3-D-hydroxybutyrate and 2-ketobutyrate were inversely associated with IR. However, phenylalanine, methionine, and cysteine could not be reliably quantified, and there was no significant association between tyrosine and Log IR. These observations suggest that metabolite-IR associations are different in insulin sensitive healthy children with normal body fat, and/or they change during childhood, depending on age, and changes in weight and IR. Many of the previously reported metabolite associations are likely to be consequences of weight gain and/or IR. The present findings are consistent with the proposal that healthy insulin sensitive children have intact BCKD activity and oxidize BCAA efficiently, whereas weight gain and IR impair BCKD activity and result in elevated BCAA.

The present study also suggests that IR is associated with several other disturbances in central energy pathways. Insulin resistance is associated with impaired metabolite flux through the Krebs cycle.[35] Consistent with this, we found that citrate was inversely associated with log IR. Decreased citrate levels may indicate a decreased contribution of fatty acids to the pool of acetyl-coA entering the Krebs cycle. We also observed that reduced 3-D-hydroxybutyrate concentrations were associated with Log IR, perhaps indicating decreased lipolysis and fatty acid oxidation. Recently, Mastrangelo et al observed reduced 3-D-hydroxybutyrate in obese prepubertal children with IR,[36] an observation perhaps attributable to hyperinsulinemia. It is possible to speculate that a reduced capacity to generate ketones might have significant long term implications for body weight regulation.

In addition, in longitudinal analysis higher alanine and lactate levels were associated with Log IR throughout childhood. Such a signature also occurs in obese prepubertal children with IR.[29] This pattern may reflect enhanced mitochondrial pyruvate oxidation secondary to reduced fatty acid oxidation under conditions of lower intramitochondrial NADH/NAD+ and acetyl-CoA. This observation also suggests IR is associated with increased flux through the Cahill and Cori cycles in the liver and muscle. In particular, subjects in the 91st centile of HOMA-IR in adolescence exhibited increasing lactate levels from 7 years of age, suggesting that elevated lactate levels might be an early precursor of IR.

Lastly, this analysis identified novel negative associations of histidine, lysine and arginine concentrations with Log IR. Interestingly, subjects in the 91st centile of HOMA-IR adolescence exhibited lower histidine levels at ages 9, 13, 14, and 15 years, which corresponded to the period where Log IR trajectories diverged between groups. When stratifying children at the 91st centile of HOMA-IR at 14 to 16-year of age, histidine levels were lower in children with higher Log IR at ages 9, 13, 14, and 15. However, the association was not significant after adjustment for BMI z-score, suggesting it is a weight-related effect. Because BMI z-scores increase from childhood to adolescence, and this is a factor influencing IR, it is not surprising that metabolic signatures are influenced by body composition. Histidine has been identified previously as a marker of IR in obese adults, with potential roles in inflammation and oxidative stress,[36] especially in obese adult women with the metabolic syndrome.[37] Histidine supplementation has also been shown to reduce IR,[38] possibly by protecting against oxidative stress in adipose tissue, stimulation of adiponectin secretion from adipocytes, and improving release of fatty acids from adipocytes during lipolysis.[38] The reason for the inverse association of histidine with IR in this study remains uncertain, but it is possible that low histidine levels might contribute to weight-related changes in IR and oxidative stress. Furthermore, the anthropometric and metabolic changes diverge between groups around the APHV, indicating puberty as a critical period shaping the molecular phenotype.

This study, as well as all of the previous studies of children reported in the literature, has certain limitations. These include

subject selection, limited sample sizes, limited phenotypic data on growth. However, a significant strength of EarlyBird is that it is a truly longitudinal study of a cohort of healthy children, with detailed annual biochemical and phenotypic measurements from age 5 to 16 years. Importantly, the majority of the children were of normal weight and the IR observed was within the normal physiological range. Therefore, the EarlyBird study is not expected to reveal the consequences of more severe obesity or IR, rather it provides insights into the beginning of these conditions. Our observations are subject to replication, especially for the BCAA results which show a negative relationship with IR. The inclusion of healthy children who were of normal weight and insulin sensitivity reduces the variance of IR in the study population, and hence differences may be more difficult to demonstrate. The HOMA method may also have certain limitations as a measure of IR, although it has been shown to correlate highly with clamp-derived measurements.[14] Finally, the duration of follow-up, although 10 years long, may not yet be sufficient to detect the long-term emergence of obesity and IR. Therefore, longer term follow-up of the cohort will provide further opportunities to examine the predictive value of these biomarkers.

In conclusion, this unique longitudinal study of healthy, predominantly normal weight, children shows that IR is associated with a characteristic molecular phenotype, including lower levels of BCAA, Krebs cycle intermediates and ketogenesis, and increased activity of Cori and Cahill cycles, while elevated lactate concentrations were found to precede IR. These findings reveal the broad functional relationship between insulin action and multiple pathways of energy metabolism during normal child growth and development. Moreover, the directions of observed associations suggest that most previously reported findings, including elevated BCAA concentrations in obese children, are consequences of IR and obesity. In the context of childhood obesity, however, impaired oxidation of BCAA, glucose and fatty acids, and reduced ketogenesis, are all likely to be maladaptive, and liable to perpetuate the obese condition and predispose to diabetes. The findings also suggest that reduced concentrations of histidine might be of pathophysiological significance. Finally, Neel postulated that normal insulin sensitivity evolved to maintain efficient metabolic homeostasis and adaptation, whereas modern-day obesity resulted in IR and hyperinsulinemia, which were maladaptive and predisposed to diabetes.[39] Our findings broadly support this hypothesis.

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## CONFLICT OF INTEREST

J.H., J.P., and A.J. are employees of Plymouth University Peninsula School of Medicine and Dentistry. F.P.M., M.K., J.H., O.C., L.D.S., and S.C. are or were employees of Nestlé Research at the time the work has been performed. J.H. and A.J. have received funding from Nestlé Research. The authors have no other dualities of interest to declare.

## AUTHOR CONTRIBUTIONS

J.H., A.J., F.P.M., L.D.S., and S.C. were involved in the acquisition of the data and F.P.M., J.H., O.C., M.K., J.H., and J.P. were involved in the analysis and interpretation of the data. J.H., J.P., and F.P.M. drafted the manuscript and all authors have read and approved the final manuscript. J.P. is guarantor of the work.

## DATA SHARING STATEMENT

Data may be available upon request to Francois-Pierre Martin and Jonathan Pinkney, subject in particular, to ethical and privacy considerations.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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