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Censoring-Aware Multielement Biomonitoring of Paediatric Scalp Hair in Spain

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Abstract

Scalp hair enables non-invasive paediatric human biomonitoring, but multielement interpretation is complicated by element-specific detectability, external contamination, analytical heterogeneity and left-censored measurements. We integrated an audited 42-element programme from archived scalp hair collected in 2001 from 120 children aged 6–9 years and 97 adolescents aged 13–16 years in Alcalá de Henares, Spain. Legacy ICP–OES and later ICP–MS participant-level records were harmonised using element-specific provenance rules, and non-detects were analysed without deterministic substitution. Primary multivariate inference used a fully observed seven-element STRICT CORE (Cr, Fe, Cu, Zn, Se, Mo and Sn) with principal component analysis, PERMANOVA and dispersion testing. Fifteen elements differed between developmental strata after Benjamini–Hochberg correction. Child–adolescent grouping was associated with 10.8% of STRICT CORE variation (PERMANOVA $p = 0.0002$), with no evidence of unequal dispersion (PERMDISP $p = 0.488$), whereas sex accounted for smaller proportions within children (2.7%) and adolescents (5.5%). Residential heterogeneity was also evident, although the adolescent pattern was dispersion-sensitive. Principal findings were highly stable across population, analytical-limit and platform sensitivities. These results identify child–adolescent grouping as the largest reproducible structured correlate examined and demonstrate how explicit censoring and analytical provenance can strengthen retrospective multielement hair biomonitoring.

Keywords: human biomonitoring; scalp hair; paediatric exposure; trace elements; potentially toxic elements; multielement analysis; left-censored data; exposure assessment

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1. Introduction

Human exposure to inorganic elements occurs as a mixture rather than as independent single-element events. This is particularly relevant during childhood and adolescence, when growth, diet, physiology, behaviour and contact with environmental media change rapidly. Essential elements, potentially toxic metals and metalloids, geogenic tracers and technology-related elements may coexist within the same biological matrix, and their covariation can reflect shared exposure pathways, biological handling and matrix-specific incorporation. Multielement approaches may therefore reveal structure that element-by-element analyses

miss [1–3]. From a toxicological and exposure-assessment perspective, this mixture-oriented view is particularly relevant in paediatric populations, in which developmental changes may influence both exposure patterns and biomarker interpretation.

Scalp hair is attractive for paediatric biomonitoring because collection is non-invasive, requires little infrastructure, permits repeated sampling and yields a keratinised matrix that can be archived; modern methods can quantify broad elemental panels from small sample masses [4,5]. Interpretation is nevertheless element-specific. Hair may reflect endogenous incorporation, external deposition or both, and its effective exposure window is not equally established across elements [6,7]. Concentrations may also vary with hair growth and characteristics, cosmetic treatment, washing, particulate deposition and analytical procedure. Consistent with this complexity, correspondence with blood or plasma is poor for several elements, whereas stronger paediatric biomarker performance has been reported for arsenic and, to a lesser extent, selenium [8,9]. Ambient particulate matter may additionally contribute residual metal(loid)s despite washing [10]. Hair-element measurements are therefore best interpreted as element-specific population-level biomonitoring signals rather than universal proxies for systemic dose, nutritional status or toxicological risk.

Analytical comparability is another major challenge. Published hair reference intervals vary substantially among populations and laboratories because of participant characteristics, geography, sample preparation, digestion, instrumentation, analytical sensitivity and treatment of measurements below analytical limits [11,12]. Digestion can affect recovery and reproducibility in ICP–MS analysis [13], making explicit element-level provenance and QA/QC essential when retrospective studies integrate different analytical campaigns. Left-censoring adds a related statistical problem: replacing non-detects with zero, LoD/2 or another constant can alter distributions, variances and associations, especially when censoring differs between groups [14–18]. Integrative analysis should therefore preserve censoring, distinguish measured from model-derived information and avoid creating an artificially complete matrix.

Developmental stage and sex can also structure hair composition. Age-associated increases in Ca, Ba, Mg, Zn and Sr and other developmental differences have been reported in paediatric populations [19–22], while sex-associated concentrations and inter-element relationships have been described for multiple elements [23–26]. Evidence for less conventionally monitored elements is more limited, although developmental and nutritional context may also influence rare earth elements [27]. These findings argue against assuming a single paediatric hair-element distribution across broad age ranges or sexes without evaluating population structure.

Residential context may add further heterogeneity. Geographical structuring of paediatric hair-element profiles has been reported in differing environmental settings [28–31]. Studies incorporating environmental matrices show, however, that relationships are element- and pathway-specific: drinking-water characteristics have been associated with hair composition, and integrated soil–crop–hair studies illustrate the value of linked environmental and biological compartments [32,33]. Residential classification alone therefore cannot establish a causal source.

The Alcalá de Henares paediatric biomonitoring programme offers an unusual opportunity to address these issues integratively. Hair collected in 2001 from well-characterised children and adolescents supported earlier studies of Pb and conventional trace elements and age-, sex- and residential-area variability [34–37]. Later analyses of archived material extended coverage to Ag [38], Ba–Sr–V [39], nutritionally relevant and mixed-source elements [40], and rare earth and technology-related elements [41], yielding an audited 42-element inventory. These publications focused on individual elements or coherent elemental families; they did not test whether the combined participant-level dataset contained a repro-

ducible multielement architecture. The present study is therefore a secondary integrative analysis rather than a re-estimation of previously reported element-specific distributions. By jointly evaluating essential, potentially toxic and emerging or technology-related elements, this integration addresses a mixture-oriented human-biomonitoring question that could not be examined in the preceding element-specific studies.

The primary objective was to determine whether the joint scalp-hair multielement profile differed between children aged 6–9 years and adolescents aged 13–16 years. Secondary objectives were to characterise sex- and residential-associated patterning, identify contributors to multivariate structure, and test robustness to historical population definitions, analytical-limit scenarios and alternative multivariate panels. Because pubertal stage was not measured, the child–adolescent contrast was treated as a developmental-stage proxy rather than a direct measure of pubertal or endocrine maturation. We hypothesised that developmental stage would be the largest reproducible correlate, with sex and residential setting contributing smaller or more context-dependent variation.

2. Materials and Methods

2.1. Study Design, Population and Ethics

This retrospective secondary analysis used archived scalp hair collected in Alcalá de Henares, Spain, in April–May 2001 from 120 children aged 6–9 years (50 boys, 70 girls) and 97 adolescents aged 13–16 years (29 males, 68 females). Participants had been recruited through schools and met predefined eligibility criteria intended to reduce major non-environmental variability in hair-element composition, including continuous residence in Alcalá de Henares and naturally dark, untreated hair; additional health- and treatment-related criteria were described previously [34–37]. Written parental/legal-guardian consent and assent where appropriate were obtained in the original study, which followed the Declaration of Helsinki. The retrospective integrative analysis was approved by the University of Alcalá CEI-EA (CEIP/2025/3/089; 5 May 2025).

The primary population retained all 217 participants with valid archived analytical information. Historical analytical populations of 117 children and 96 adolescents were reproduced as prespecified sensitivity analyses rather than treated as evidence of current ineligibility.

2.2. Study Area and Residential Classification

The historical programme classified Alcalá de Henares *a priori* into four broad residential/environmental zones: Zone I, lower-density residential areas with more green space; Zone II, compact urban/residential areas; Zone III, areas characterised by major road traffic; and Zone IV, a heterogeneous mixed residential–industrial setting [34,36]. These categories were retained as ecological residential classifications, not individual exposure measures or source-attribution variables. Children were represented in Zones I, II and IV and adolescents in all four zones; residential analyses were therefore stratified by developmental group.

2.3. Scalp-Hair Collection and Archived Material

Hair was cut from the occipital region as close to the scalp as practicable, and the proximal fraction (approximately 1–2 cm for longer hair) was retained in clean paper envelopes under dry archival conditions. All specimens originated from the 2001 sampling campaign; later dates refer only to subsequent analytical processing of archived hair and not to a second population sampling event.

Two analytical eras contributed to the integrated dataset: historical ICP–OES measurements and later ICP–MS measurements on remaining archived material. Each element was

assigned one audited participant-level analytical source, and measurements from different campaigns were not averaged.

2.4. Analytical Campaigns and Quality Assurance

The integrated 42-element programme combined legacy ICP–OES and four later ICP–MS campaigns. Historical ICP–OES measurements were generated using a Thermo Jarrell Ash ICAP 61 (Thermo Jarrell Ash Corporation, Franklin, MA, USA), with TORT-2 certified reference material and element-specific historical analytical limits retained as originally documented. The published legacy elemental panels differed between developmental strata. The later campaigns—Ag, Ba–Sr–V, REE/technology-related elements, and a nutritionally relevant/mixed-source panel—used a PerkinElmer NexION 350D ICP–MS (PerkinElmer, Waltham, MA, USA) at CCiTUB, Universitat de Barcelona. Importantly, identical hair decontamination and sample-preparation protocols were employed across all analytical campaigns, following the procedure previously described by Peña-Fernández et al. [34]. Briefly, hair samples were washed with a 1% (*v*/*v*) Triton X-100 solution (Sigma-Aldrich Co., St. Louis, MO, USA) prepared with ultrapure Milli-Q® water (Merck Millipore, Darmstadt, Germany) for 5 min in an ultrasonic bath (J.P. Selecta Ultrasons, Abrera, Barcelona, Spain). A second washing step was performed using ultrapure Milli-Q® water without detergent, followed by thorough rinsing with ultrapure water. The cleaned samples were subsequently dried at 50 °C to constant weight and digested using 65% HNO_3 (Suprapur®, Merck, Darmstadt, Germany). The differences between analytical campaigns concerned the timing of sample digestion and analysis and the instrumental techniques employed, with the subsequent ICP–MS determinations performed under collision-cell conditions. Campaign-specific external calibration, internal-standard correction where applicable, procedural blanks and certified/reference materials were used, with reported recoveries generally within approximately 90–110% for later ICP–MS campaigns and 78–110% for the historical ICP–OES programme [42–45].

Because Ni and V were not included in the published adolescent legacy panel, the primary participant-level Ni and V fields for both developmental strata were derived from the later ICP–MS campaign, preventing developmental stage from being confounded with analytical platform for these elements.

Historical analytical limits were retained from the original documentation. For later multielement ICP–MS datasets, primary LoDs were derived from procedural-blank variability as 3σ where applicable; the published cohort-specific Ag LoDs were retained. A separate instrument-file "orientative" threshold was used only as a documentary sensitivity scenario and was not treated as equivalent to the primary LoD [46]. Detailed campaign-specific sample preparation, calibration, internal standards, blanks, certified/reference materials, recoveries, LoDs/LoQs and element-level provenance are provided in Supplementary Methods SM1–SM3 and Supplementary Table S1.

2.5. Participant-Level Harmonisation and Historical Data Reconciliation

Source files were cross-audited, participant identifiers were harmonised, and each element received a canonical concentration field, censoring indicator and censoring limit. Duplicate analytical records were not treated as independent observations, and harmonisation decisions were documented according to general principles for retrospective data integration [47].

Historical numerical substitutions were not assumed to represent measured concentrations. Reconstruction was permitted only when the archived source and historical reporting convention could be reconciled one-to-one. For example, 81 historical zero-coded child Ti records were reconstructed as left-censored at the documented 0.02 $\mu g\ g^{-1}$ LoD

because the remaining 39 positive values exactly reproduced the historically reported detections. By contrast, adolescent Al and Pb, historical Mn, and missing adolescent participant-level Be and Tl could not be reconciled with sufficient row-level certainty and were gated from affected analyses rather than reconstructed through undocumented assumptions. No synthetic participant-level values were created. Full reconciliation rules are given in Supplementary Methods SM4.

2.6. Left-Censored Data and Descriptive Estimation

Detected observations retained their measured concentration with censored = FALSE; non-detects retained the applicable limit with censored = TRUE. Deterministic substitution by zero, LoD/2, LoD/$\sqrt{2}$ or the LoD itself was not used for primary inference [14,15,17,48]. Fully quantified elements were described directly, whereas left-censored elements were analysed using NADA/NADA2 procedures, including reverse Kaplan–Meier, regression on order statistics or lognormal censored maximum-likelihood estimation where prespecified estimability criteria were met [16,18,49,50]. Elements with >80% censoring were treated primarily as detectability outcomes rather than forced into continuous distributional estimation.

Model-derived Q1, median and Q3 estimates were additionally subjected to a deterministic censoring-bound audit. For a target quantile probability p, if the cumulative proportion known to lie at or below censoring threshold L was at least p, the corresponding quantile was constrained to $Q_p \leq L$. With multiple LoDs, the smallest threshold satisfying that condition defined the bound. Estimates incompatible with this information were retained for audit only and reported as not point-estimable (NE); no $\log_2$ ratio was calculated from a gated quantile. This rule affected descriptive effect-size reporting only and did not alter Peto–Peto tests, FDR control or STRICT CORE multivariate inference.

2.7. Element-Specific and Adjusted Inference

The primary developmental comparison contrasted children with adolescents. Left-censored outcomes were tested using the Peto–Peto generalised Wilcoxon procedure in NADA2 and fully quantified outcomes using Wilcoxon rank-sum tests; Benjamini–Hochberg FDR correction was applied across the predefined developmental family [51]. Sex analyses were performed separately within each developmental stratum. Residential-zone effects were likewise evaluated within strata; uncensored multi-zone outcomes used Kruskal–Wallis tests, followed, where appropriate, by BH-adjusted pairwise comparisons.

NADA2 censor-aware permutation tests were prespecified as methodological sensitivities rather than alternative significance-seeking analyses; discordant FDR classifications were labelled method-sensitive. Complementary lognormal censored-regression models (NADA cenreg) evaluated developmental stage adjusted for sex, with adolescent-to-child ratios obtained by exponentiating the developmental coefficient. A secondary common-support model additionally included residential zone, restricted to Zones I, II and IV. These models were supportive rather than replacements for the primary analyses. Complete results are provided in Supplementary Table S2.

2.8. Observed-Only STRICT CORE and Multivariate Analyses

Primary multivariate inference was deliberately restricted to a fully observed STRICT CORE to prevent imputed or model-derived non-detects from generating apparent group separation. Eligibility required complete participant coverage, zero censoring, finite positive concentrations and no unresolved historical gate. Seven elements met these criteria: Cr, Fe, Cu, Zn, Se, Mo and Sn. Cr, Cu, Zn and Sn derived from historical ICP–OES and Fe, Se and Mo from later ICP–MS, but each element used the same designated platform in children and adolescents.

STRICT CORE concentrations were $\log_{10}$-transformed, centred and scaled before PCA. Stability of PC1/PC2 loadings was evaluated in 1000 bootstrap resamples, allowing component interchange and sign reversal before concordance assessment. PERMANOVA used Euclidean distances on the same transformed-standardised matrix with 4999 permutations [52] and was paired with PERMDISP because distance-based tests may reflect multivariate location, dispersion or both [53,54]. Developmental stage was assessed in the pooled population; sex and residential zone were tested within developmental strata.

Wider 12-element CORE and 16-element EXPANDED panels were retained only as sensitivities. Where censor completion was required, ROS was fitted to the pooled age dataset rather than separately by group. Multiple factor analysis was a secondary platform-integration exercise and did not contribute to primary inference.

2.9. Sensitivity Analyses and Robust Element Networks

Prespecified sensitivities reproduced the historical 117-child and 96-adolescent populations, alternative analytical-limit scenarios, the censor-aware permutation procedure, and a later-ICP–MS-only developmental analysis. Robustness was assessed through preservation of effect direction and FDR classification, changes in effect estimates, Spearman concordance, logical quantile-bound validation, PCA/bootstrap stability, and concordance among STRICT CORE, CORE and EXPANDED distance structures (Supplementary Table S3).

Secondary element networks used observed STRICT CORE concentrations only. Within each developmental stratum, Kendall's τ associations were retained when BH $q \leq 0.05$, $|\tau| \geq 0.25$, bootstrap sign stability was ≥80% across 1000 resamples and the 95% bootstrap interval excluded zero. No community structure was inferred because stable-module analysis was not prespecified.

2.10. Software and Reproducibility

Analyses were performed in R 4.4.2 [55] using NADA 1.6-1.2 [49], NADA2 2.0.2 [50], *survival* 3.7-0 [56], *vegan* 2.7-2 [57], *FactoMineR* 2.13 [58] and *igraph* 2.1-4 [59]. Random seeds were fixed for permutation and bootstrap procedures. The frozen analysis package contains the harmonised dataset, decision logs, manifests, scripts, outputs and session information.

3. Results

3.1. Study Population and Integrated Analytical Architecture

The final primary population comprised 120 children and 97 adolescents. Children included 50 boys and 70 girls and were distributed across Zone I ($n = 24$), Zone II ($n = 60$) and Zone IV ($n = 36$). Adolescents included 29 males and 68 females and were distributed across Zone I ($n = 36$), Zone II ($n = 20$), Zone III ($n = 24$) and Zone IV ($n = 17$).

Across the historical and later analytical campaigns, the audited programme encompassed 42 distinct elements. Analytical coverage and censoring varied substantially among elements, precluding their treatment as an equivalent set of continuous variables. Highly censored elements were retained principally as detectability information, historically ambiguous participant-level fields were gated, and only elements with complete uncensored measurements were admitted to the primary STRICT CORE multivariate analysis.

3.2. Developmental-Stage-Associated Elemental Fingerprint

Primary developmental-stage testing identified 15 elements that remained significant after BH-FDR correction (Table 1, Figure 1). Among elements with censor-aware point-estimable medians, adolescents had higher concentrations of Mg, Zn, Ca, Sr, Sn and Ba than children. Median Mg increased from 9.004 to 23.98 μg g^{-1} ($\log_2$ adolescent/child ratio = 1.41), Ca from 168.1 to 325.3 μg g^{-1} ($\log_2$ ratio = 0.95), Sr from 0.4115 to 1.105 μg g^{-1}

($\log_2$ ratio = 1.42), Zn from 110.9 to 144.0 µg g^{-1}, Sn from 1.233 to 1.553 µg g^{-1} and Ba from 0.1933 to 0.2866 µg g^{-1}.

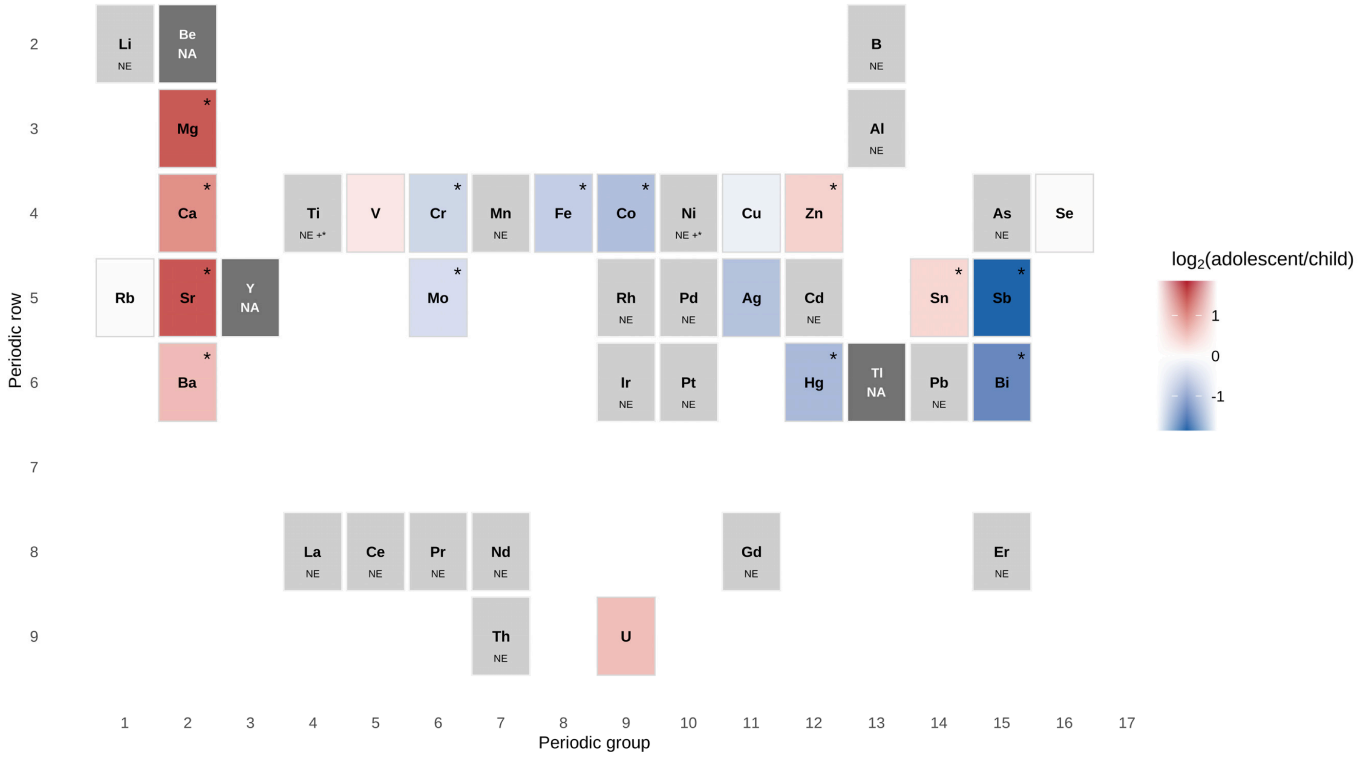


Figure 1. Developmental-stage periodic fingerprint across the audited 42-element scalp-hair programme. Elements are positioned according to periodic table group and row. Colour represents the $\log_2$(adolescent/child) median ratio where a censor-compatible numerical estimate was available; red indicates higher values in adolescents and blue lower values. Asterisks indicate elements meeting the Benjamini–Hochberg false-discovery-rate criterion ($q < 0.05$) in the primary developmental comparison. NE denotes a numerical effect estimate that was not point-estimable under the prespecified censoring-bound rule; for NE elements, "+" indicates an inferential direction towards higher concentrations or detectability in adolescents. NA denotes an element not available for the integrated developmental comparison. Children, $n = 120$; adolescents, $n = 97$.

Conversely, adolescents showed lower concentrations of Sb, Cr, Fe, Hg, Bi, Mo and Co. The largest proportional contrasts included Sb, which decreased from 0.0331 to 0.0092 µg g^{-1} ($\log_2$ ratio = −1.85), and Bi, which decreased from 0.0059 to 0.0023 µg g^{-1} ($\log_2$ ratio = −1.38). Median Cr decreased from 0.6429 to 0.4693 µg g^{-1}, Fe from 7.544 to 5.165 µg g^{-1}, Hg from 0.7632 to 0.4389 µg g^{-1}, Mo from 0.0298 to 0.0231 µg g^{-1} and Co from 0.0062 to 0.0038 µg g^{-1}.

Ni and Ti also showed significant developmental-stage differences, with the inferential direction indicating higher concentrations or detectability in adolescents. However, 51.7% of child Ni and 67.5% of child Ti observations were censored. Their child medians were therefore not point-estimable under the prespecified censoring-bound rule, and numerical $\log_2$ fold changes were not reported.

Table 1. Element-specific developmental-stage differences surviving Benjamini–Hochberg false-discovery-rate correction.

Element	Child Censored (%)	Adolescent Censored (%)	Child Median ($\mu g\ g^{-1}$)	Adolescent Median ($\mu g\ g^{-1}$)	$\log_2(A/C)$	Direction	BH-Adjusted q	Point-Estimate Status	Method-Sensitive
Mg	7.5	0.0	9.004	23.98	1.41	Higher in adolescents	7.77×10^{-19}	Point-estimable	No
Ca	46.7	2.1	168.1	325.3	0.95	Higher in adolescents	1.61×10^{-9}	Point-estimable	No
Sr	23.3	37.1	0.4115	1.105	1.42	Higher in adolescents	2.92×10^{-5}	Point-estimable	No
Ba	38.3	35.1	0.1933	0.2866	0.57	Higher in adolescents	0.0022	Point-estimable	No
Zn	0.0	0.0	110.9	144.0	0.38	Higher in adolescents	1.54×10^{-18}	Point-estimable	No
Sn	0.0	0.0	1.233	1.553	0.33	Higher in adolescents	0.0022	Point-estimable	No
Ni	51.7	43.3	NE	0.1687	NE	Higher in adolescents	2.50×10^{-5}	Not point-estimable under censoring	No
Ti	67.5	0.0	NE	0.8555	NE	Higher in adolescents	7.33×10^{-26}	Not point-estimable under censoring	No
Sb	0.8	1.0	0.0331	0.0092	−1.85	Lower in adolescents	2.25×10^{-21}	Point-estimable	No
Bi	42.5	60.8	0.0059	0.0023	−1.38	Lower in adolescents	4.20×10^{-6}	Point-estimable	No
Hg	1.7	2.1	0.7632	0.4389	−0.80	Lower in adolescents	2.68×10^{-6}	Point-estimable	No
Co	25.8	23.7	0.0062	0.0038	−0.72	Lower in adolescents	0.0103	Point-estimable	Yes
Fe	0.0	0.0	7.544	5.165	−0.55	Lower in adolescents	2.01×10^{-11}	Point-estimable	No
Cr	0.0	0.0	0.6429	0.4693	−0.45	Lower in adolescents	2.02×10^{-12}	Point-estimable	No
Mo	0.0	0.0	0.0298	0.0231	−0.37	Lower in adolescents	9.83×10^{-6}	Point-estimable	No

$N = 120$ children and $N = 97$ adolescents. A/C = adolescent/child. NE = not point-estimable under censoring. For Ni and Ti, the primary Peto–Peto inference and effect direction are retained, but the child median and derived $\log_2(A/C)$ ratio are NE because a modelled median would violate the logical censoring bound. Co is method-sensitive because the primary Peto–Peto and prespecified censor-aware permutation sensitivity differ in FDR classification. Ag, Rb and V are also method-sensitive but do not appear here because they do not meet the primary developmental BH-FDR criterion.

Co satisfied the primary BH-FDR criterion but was classified as method-sensitive because the prespecified censor-aware permutation analysis did not reproduce the primary Peto–Peto classification. Ag, Rb and V showed the converse pattern: their permutation sensitivities met the FDR criterion whereas their primary Peto–Peto tests did not. These discrepancies were retained explicitly rather than resolved by selecting the more favourable analytical method.

Complementary censored-regression models substantially supported the unadjusted developmental pattern. After adjustment for sex, the developmental-stage coefficient remained BH-FDR significant for 16 elements: the 15 identified in the primary analysis plus Cu (Supplementary Table S2). For Cu, the sex-adjusted adolescent-to-child concentration ratio was 0.779 (BH-adjusted $q = 0.0128$), corresponding to an estimated 22.1% lower concentration in adolescents after adjustment for sex, whereas the primary unadjusted comparison was not statistically significant after FDR correction ($q = 0.2215$). This difference may partly reflect the unequal sex distribution between developmental groups, particularly because females exhibited higher Cu concentrations within both strata. However, the association was attenuated after additional adjustment for residential zone within the common geographical support (ratio = 0.860; BH-adjusted $q = 0.2074$), indicating that the adjusted Cu association should be interpreted cautiously.

3.3. Observed-Only STRICT CORE Multielement Structure

The observed-only STRICT CORE comprised Cr, Fe, Cu, Zn, Se, Mo and Sn and contained complete measurements for all 217 participants. Because no censoring or imputation was present, the primary multivariate analysis was independent of assumptions concerning completion of non-detects.

The first two principal components explained 43.55% of total standardised multielement variance, with PC1 accounting for 24.11% and PC2 for 19.43%; inclusion of PC3 increased the cumulative variance explained to 59.54%. PC1 was characterised principally by negative loadings for Fe (−0.552), Cr (−0.492) and Mo (−0.451) and a positive loading for Zn (0.406). PC2 was driven positively by Cu (0.592), Zn (0.410) and Se (0.331), with negative contributions from Sn (−0.417) and Mo (−0.324) (Figure 2).

Bootstrap analysis supported the stability of the dominant PCA axes. Across 1000 resamples, the median loading-vector Spearman correlation with the original solution was 0.929 for PC1 and 0.893 for PC2, while the median bootstrapped variance contributions were approximately 25.4% and 19.7%, respectively.

Developmental stage was strongly associated with the joint STRICT CORE structure, explaining 10.77% of multivariate variation (PERMANOVA $R^2 = 0.1077$, $p = 0.0002$). Multivariate dispersion did not differ between children and adolescents (PERMDISP $p = 0.488$), supporting interpretation of the developmental-stage signal primarily as a difference in multivariate location rather than unequal within-group dispersion.

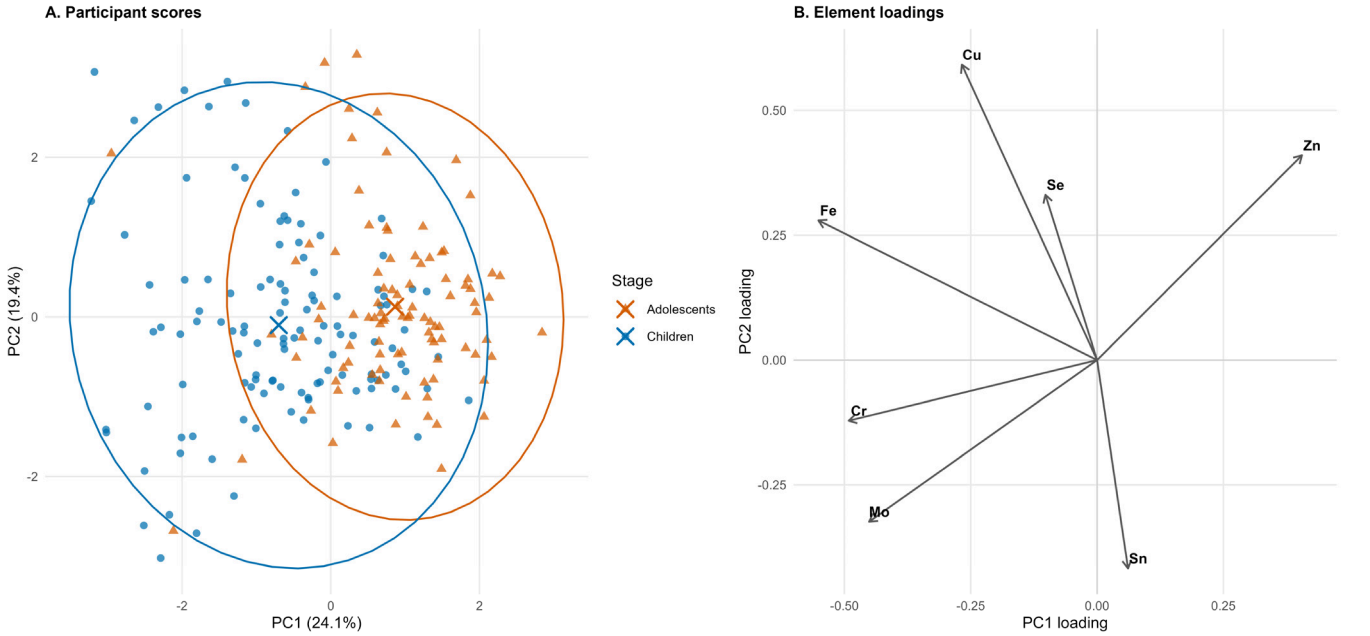


Figure 2. Principal component analysis of the observed-only STRICT CORE multielement profile. The STRICT CORE comprised Cr, Fe, Cu, Zn, Se, Mo and Sn and contained complete uncensored measurements for all 217 participants. Concentrations were $\log_{10}$-transformed, centred and scaled before analysis. (**A**) Participant scores for children ($n = 120$) and adolescents ($n = 97$) on PC1 and PC2; large crosses indicate group centroids and ellipses provide a visual summary of the group score distributions. (**B**) Element loadings on PC1 and PC2. PC1 and PC2 accounted for 24.1% and 19.4% of total standardised multielement variance, respectively.

3.4. Sex-Associated Multielement Patterning

Sex explained a smaller but detectable component of STRICT CORE multielement variation within each developmental stratum. Among children, sex accounted for 2.71% of variation (PERMANOVA $R^2 = 0.0271$, $p = 0.0022$), without evidence of unequal dispersion (PERMDISP $p = 0.242$). Among adolescents, the corresponding effect was larger, explaining 5.51% of variation ($R^2 = 0.0551$, $p = 0.0002$), again without evidence of dispersion heterogeneity (PERMDISP $p = 0.623$).

Element-specific analyses identified 12 BH-FDR-significant sex-associated elements in each developmental stratum (Supplementary Table S2; Figure 3A,B). In children, point-estimable concentrations of Ca, Sr, Mg, Cu, Ba, Al, Ni, Co, U, Ag and Fe were higher in girls, whereas Sb was lower. Some of the largest proportional contrasts involved Ca, Sr, Mg, Ba, Al and Ni.

Among adolescents, females showed higher point-estimable concentrations of Zn, Cu, Fe, Co, Ag, V, U, Sn, Ni and Ca. Sr showed an inferential direction consistent with higher concentrations in females, although one sex-specific central estimate was not point-estimable under the censoring rule. Rb also met the BH-FDR criterion but did not support a stable direction based on point-estimable central quantiles.

Sex-associated elemental patterning was therefore widespread at the individual-element level but accounted for substantially less observed multivariate variation than developmental stage.

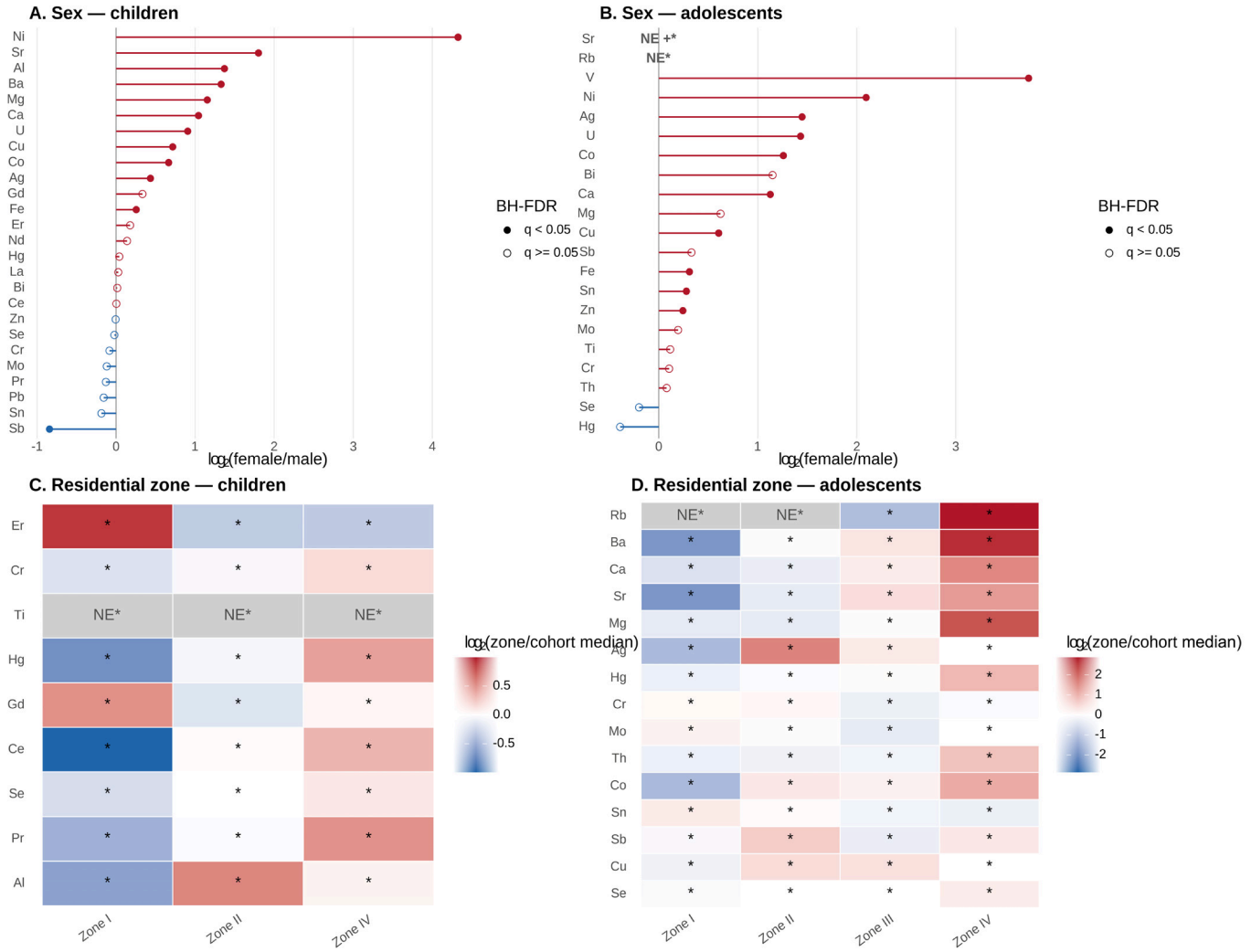


Figure 3. Sex- and residential-associated elemental patterning within developmental strata. (**A**) Sex-associated effects in children ($n = 120$; 50 boys and 70 girls). (**B**) Sex-associated effects in adolescents ($n = 97$; 29 males and 68 females). Effects are expressed as $\log_2$(female/male); positive values indicate higher concentrations in females and negative values higher concentrations in males. Filled markers denote BH-FDR $q < 0.05$ and open markers $q \geq 0.05$. NE denotes an effect that was not numerically point-estimable under the censoring-bound rule; where shown, "+" indicates an inferential direction towards higher values in females, and an asterisk denotes BH-FDR significance. (**C**) Residential-zone patterning in children: Zone I, $n = 24$; Zone II, $n = 60$; Zone IV, $n = 36$. (**D**) Residential-zone patterning in adolescents: Zone I, $n = 36$; Zone II, $n = 20$; Zone III, $n = 24$; Zone IV, $n = 17$. Heat-map colours represent $\log_2$(zone/within-stratum cohort median), with positive values indicating concentrations above and negative values below the corresponding developmental-stratum median. Asterisks in panels (**C**,**D**) denote elements with a BH-FDR-significant overall residential-zone effect and should not be interpreted as pairwise significance for an individual zone; pairwise post hoc comparisons are reported in Supplementary Table S2. NE denotes a zone-specific central estimate that was not point-estimable under the prespecified censoring rule.

3.5. Residential-Zone Heterogeneity

Residential setting was associated with multielement structure within both developmental strata, although the nature of the association differed (Figure 3C,D).

Among children, residential zone explained 4.89% of STRICT CORE variation (PERMANOVA $R^2 = 0.0489$, $p = 0.0002$), with no evidence of unequal dispersion (PERMDISP $p = 0.383$). Nine elements showed BH-FDR-significant overall zone differences: Er, Cr, Ce, Gd, Hg, Ti, Se, Pr and Al. Directional summaries indicated higher Cr, Ce, Hg, Pr and Se in

Zone IV than in Zone I, whereas Al was highest in Zone II. Ti was interpreted principally through detectability because of substantial censoring.

Among adolescents, residential zone explained 10.02% of STRICT CORE variation ($R^2 = 0.1002$, PERMANOVA $p = 0.0002$). PERMDISP was also significant ($p = 0.0264$), indicating that the residential result was dispersion-sensitive and should not be interpreted as a simple difference in multivariate centroids.

Fifteen adolescent elements showed BH-FDR-significant overall zone effects: Rb, Ba, Ca, Sr, Mg, Ag, Hg, Cr, Mo, Co, Th, Sn, Sb, Cu and Se. Ba, Ca, Mg and Sr showed their highest central concentrations in Zone IV, whereas Cr and Mo were highest in Zone I and lowest in Zone III. Other elements displayed distinct patterns, indicating that the residential signal did not follow a single common gradient. Pairwise censor-aware post hoc comparisons are reported in Supplementary Table S2.

3.6. Robustness to Population, Analytical-Limit and Methodological Decisions

Primary developmental findings were highly stable in relation to historical population restrictions (Supplementary Table S3; Supplementary Figure S1). Restricting children from $N = 120$ to the historical $N = 117$ population preserved 100% of comparable effect directions and 100% of BH-FDR classifications, with Spearman concordance of effect estimates of $\rho = 0.9965$. Restricting adolescents from $N = 97$ to the historical $N = 96$ population preserved 100% of BH-FDR classifications and yielded $\rho = 1.000$; 95.2% of directly comparable effect directions were preserved. Applying both historical restrictions simultaneously produced 100% concordance in direction and BH-FDR status, with an effect-estimate correlation of $\rho = 0.998$.

Alternative analytical-limit definitions produced similarly stable results. Under the separate documentary instrument-file threshold, 100% of comparable effect directions and BH-FDR classifications were preserved, with $\rho = 0.990$ relative to the primary analysis. Individually reconstructed procedural-blank 3σ limits likewise preserved 100% of comparable directions and BH-FDR classifications, with $\rho = 0.935$.

A platform-restricted sensitivity analysis using only later ICP–MS variables likewise showed complete concordance for the 14 directly comparable point-estimable effects, preserving 100% of effect directions and BH-FDR classifications (Spearman $\rho = 1.000$).

Multivariate sensitivity analyses extending beyond the observed-only STRICT CORE also retained substantial structural agreement. Participant-level distance structure correlated at $\rho = 0.814$ between STRICT CORE and the pooled-ROS CORE sensitivity and at $\rho = 0.908$ between the CORE and EXPANDED panels. These sensitivity panels were not used to redefine the primary STRICT CORE inference.

Ag, Co, Rb and V were formally retained as method-sensitive because their primary Peto–Peto and prespecified permutation analyses differed in BH-FDR classification; no finding was reclassified according to whichever method produced the smaller p-value.

3.7. Robust STRICT CORE Element Networks

The conservative network analysis retained four robust associations among the 21 possible STRICT CORE element pairs (Supplementary Figure S2). In children, Fe was positively associated with Cu (Kendall's $\tau = 0.261$, $q = 0.000165$) and Mo ($\tau = 0.300$, $q = 0.000025$), whereas Zn and Mo were negatively associated ($\tau = -0.266$, $q = 0.000165$). In adolescents, the only robust retained association was the positive Fe–Cu relationship ($\tau = 0.277$, $q = 0.00122$).

All four retained edges showed 100% bootstrap sign stability and 95% bootstrap intervals excluding zero. No network community structure was inferred.

4. Discussion

4.1. Developmental Stage Is the Largest Reproducible Structured Correlate

The principal finding of this integrative analysis was that the predefined child–adolescent contrast represented the largest reproducible structured correlate of the scalp-hair multielement profile among the primary factors examined. Developmental stage accounted for 10.8% of variation in the observed-only STRICT CORE, whereas sex explained smaller proportions within children and adolescents. The developmental association was supported by PERMANOVA, was not accompanied by unequal multivariate dispersion, and remained stable across historical population restrictions, alternative analytical-limit scenarios and platform-restricted sensitivity analyses. Importantly, the principal multivariate analysis used only complete observed measurements, so the detected separation cannot be attributed to completion or imputation of non-detects.

This extends previous element-specific analyses of the Alcalá cohort by showing that developmental differences are expressed jointly across several elements rather than as isolated concentration shifts. The result should nevertheless be interpreted as an association with developmental grouping rather than evidence that chronological age directly determines hair-element concentrations. Children and adolescents differ simultaneously in growth, physiology, nutritional requirements, behaviour, environmental contact and potentially hair characteristics. Pubertal stage and endocrine markers were not measured; "developmental stage" therefore denotes the structured contrast between the two predefined age strata rather than a direct measurement of pubertal maturation.

The magnitude of the multivariate effect also requires appropriate context. An R^2 of approximately 0.11 does not imply that developmental stage explains most individual variability: almost 90% of standardised STRICT CORE variation remains attributable to inter-individual heterogeneity, other measured or unmeasured determinants, and residual analytical variation. Rather, developmental grouping contributed a coherent signal detectable against a heterogeneous background. This is consistent with mixture-oriented biomonitoring, where modest variance components may represent reproducible population structure without implying strong individual-level prediction [1,2]. Longitudinal studies spanning childhood and adolescence will be required to distinguish maturation itself from correlated changes in diet, behaviour and environmental exposure.

4.2. Developmental Element Patterns and Biological Interpretation

The element-specific fingerprint provides biological and exposure-related context for the multivariate result. Adolescents showed higher point-estimable hair concentrations of Ca, Mg, Zn, Sr, Sn and Ba, while Ni and Ti showed inferential evidence of higher values or detectability despite non-estimable child medians. The parallel Ca–Mg–Sr–Ba pattern is notable because Paschal et al. [19] reported increasing hair Ca, Ba, Mg, Zn and Sr through childhood towards approximately 12–14 years of age, while Długaszek and Skrzeczanowski [20] also described developmental differences involving Ca, Mg and Zn. Agreement across independent populations supports a systematic developmental component, although it does not establish a common mechanism.

Ca, Mg, Sr and Ba can co-vary within human hair. Associations among Ca, Mg and Sr and broader alkaline-earth groupings have been reported previously [25,60], and multivariate analysis in young participants has likewise identified alkaline-earth elements as important contributors to variation in hair composition [26]. Such covariance cannot distinguish endogenous regulation from shared dietary or environmental inputs, common incorporation behaviour or residual external deposition. Studies incorporating environmental matrices have, for example, reported element-specific relationships between drinking-water composition and hair Ca or Sr [32]. The present pattern is therefore best interpreted as an

alkaline-earth-rich component of the developmental hair profile rather than evidence of greater systemic mineral stores in adolescents.

Zn also increased in adolescents and loaded in the opposite PC1 direction from Fe, Cr and Mo. Age-associated increases in hair Zn have been described previously [19,20], but paediatric reference studies and systematic reviews show substantial between-population variation in absolute hair concentrations [12,22]. Diet, growth, hair characteristics, environmental deposition and analytical procedure may all contribute. Accordingly, Zn forms part of the developmental multielement pattern identified here but should not be interpreted directly as a marker of nutritional status.

Conversely, adolescents had lower hair Sb, Cr, Fe, Hg, Bi, Mo and Co. Age-dependent hair Fe has been reported previously [20], whereas developmental information for Bi, Sb and several other ultra-trace or technology-related elements remains comparatively sparse. This contrast in the literature is important: conventional essential and toxic elements have decades of comparative data, while emerging elements are only beginning to be characterised in paediatric populations. Evidence that REE accumulation can vary with age and nutritional context in children and adolescents from other settings [27] further argues for population-specific validation rather than extrapolation from adults or conventional metals.

Ni and Ti illustrate why inferential significance should be separated from numerical effect magnitude under substantial censoring. Both showed evidence of higher concentrations or detectability in adolescents, but 51.7% of child Ni and 67.5% of child Ti measurements were censored. Reporting substituted medians would have introduced unsupported numerical precision. The censoring-bound procedure instead retained the inferential direction while withholding a fold change that was not identifiable from the observed censoring structure. Co illustrates a different uncertainty: it met the primary Peto–Peto FDR criterion but not the prespecified permutation sensitivity and was therefore retained transparently as method-sensitive rather than classified according to the more favourable procedure.

Importantly, statistical significance should not be equated with biological or toxicological relevance. Although Ca, Mg, Zn and Fe are essential elements involved in numerous physiological processes, their concentrations in scalp hair cannot independently establish nutritional adequacy, deficiency or systemic mineral status [7–9]. Similarly, differences in Sb, Cr, Bi and other potentially toxic elements require cautious interpretation because the validity of scalp hair as an exposure biomarker varies considerably among elements [7–9]. Mercury represents an important exception: total Hg in scalp hair is a well-established biomarker of longer-term methylmercury exposure, particularly in populations where fish and seafood consumption is the predominant exposure pathway [7,61]. Nevertheless, the present study did not include mercury speciation or sufficient individual dietary information to establish the relative contribution of methylmercury to total hair Hg concentrations. Therefore, although the observed developmental differences in Hg may reflect variations in exposure, they cannot independently establish differences in toxicological risk or clinical effects. More generally, no element-specific health-risk assessment based on hair concentrations was undertaken, and individual clinical outcomes or paired blood and urine measurements were unavailable. Consequently, the observed elemental differences should be interpreted primarily as population-level biomonitoring associations, with element-specific consideration of biomarker validity, rather than as direct evidence of beneficial or adverse health effects.

4.3. Sex Is a Smaller but Reproducible Correlate of Multielement Variation

Sex was associated with the STRICT CORE profile within both developmental strata, although its contribution was smaller than that of developmental grouping: 2.7% of multivariate variation among children and 5.5% among adolescents. The larger adolescent

component is compatible with increasing biological and behavioural differentiation during this developmental period, but the cross-sectional design cannot identify its mechanism. Tanner stage, circulating hormones and other direct measures of pubertal maturation were unavailable and should not be inferred from the statistical sex contrast.

At the element level, the predominant pattern was towards higher hair concentrations in females. In children this involved Ca, Sr, Mg, Cu, Ba, Al, Ni, Co, U, Ag and Fe, whereas adolescent females showed higher Zn, Cu, Fe, Co, Ag, V, U, Sn, Ni and Ca, with additional censored-data evidence involving Sr and Rb. Similar sex-associated differences have been reported in human hair. Tamburo et al. [23], in a multi-site study of 943 adolescents, identified sex as an important determinant and reported higher female concentrations for several elements, including Sr, Zn and Ni. Broad multielement sex differences have also been described by Zaichick and Zaichick [24], Avino et al. [26], Długaszek and Skrzeczanowski [20], and Kusanagi et al. [21].

Sex may also influence inter-element covariance. Chojnacka et al. [25] reported sex-specific correlation structures in human hair, and recent multivariate work likewise indicates that sex can contribute to separation of elemental profiles [3]. These observations are consistent with our univariate and multivariate findings but do not imply a single biological mechanism. Hair growth and morphology, dietary behaviour, environmental contact, and physiological handling may contribute differently across elements and populations.

The original Alcalá recruitment criteria increased internal comparability by restricting inclusion to naturally dark, untreated hair and excluding relevant treatment- or health-related confounders. These criteria do not eliminate sex-associated differences in hair biology or behaviour and may also narrow generalisability to populations with different hair phenotypes or cosmetic practices. More broadly, systematic reviews support accounting for age, sex, population characteristics and analytical methodology when establishing hair reference distributions [12].

4.4. Residential Setting Is Associated with Heterogeneous Multielement Profiles

Residential setting contributed additional structure, but its interpretation differed markedly between developmental strata. Among children, residential zone explained approximately 4.9% of STRICT CORE variation without evidence of heterogeneous dispersion, supporting a modest location-associated shift within the historical ecological classification. Element-specific results were nevertheless heterogeneous: Cr, Ce, Hg, Pr and Se were higher in Zone IV than in Zone I, whereas Al was highest in Zone II and other elements followed different patterns. This argues against treating residential zone as a single exposure gradient.

Among adolescents, the nominal zone effect was larger, explaining approximately 10.0% of STRICT CORE variation, but PERMDISP was also significant. Because distance-based multivariate tests can respond to differences in centroid location, within-group dispersion or both [53,54], the adolescent result is more appropriately interpreted as residential multielement heterogeneity than as a pure shift in group centroids. Reporting only the small PERMANOVA p-value would overstate the specificity of the spatial inference.

Adolescents showed significant residential heterogeneity for Rb, Ba, Ca, Sr, Mg, Ag, Hg, Cr, Mo, Co, Th, Sn, Sb, Cu and Se. Ba, Ca, Mg and Sr reached their highest central values in Zone IV, whereas Cr and Mo followed a different pattern. Children similarly showed element-specific rather than uniform spatial contrasts. The lack of a common directional gradient further argues against treating the historical zones as a continuum of exposure intensity.

Previous hair-biomonitoring studies have also identified geographical structuring, although implicated elements and patterns vary by setting [28–31]. Studies incorporating

environmental matrices illustrate why source attribution requires additional evidence: Rosborg et al. [32] identified element-specific water–hair relationships, while Ma et al. [33] used integrated soil–crop–hair analysis to investigate environmental transfer. No equivalent participant-linked pathway measurements were available here. The present residential findings should therefore be interpreted as spatial heterogeneity within the historical cohort, not as evidence that traffic, industry, soil, water or diet was the causal source. Future studies should integrate hair with contemporaneous soil, household dust, drinking-water and dietary measurements and, where appropriate, source-apportionment or isotopic approaches.

4.5. Why Censoring and Analytical Provenance Matter

A central methodological feature of this study is that detectability was treated as information rather than as an inconvenience to be removed before analysis. Across the 42-element programme, censoring ranged from zero to almost complete non-detection. Fixed substitution by zero, LoD/2 or another constant can alter distributional properties, ranks and associations, particularly when censoring is substantial or differs between comparison groups [14–17]. Empirical work using datasets for which censored observations were known has additionally shown deterioration of distributional estimation under high censoring [18]. Preserving element-specific censoring limits and indicators therefore avoided converting unresolved observations into artificial measured concentrations.

The logical quantile-bound audit provided an additional safeguard. Censor-aware models may return numerical estimates even when directly observed censoring imposes a stricter bound on a quantile. When more than half of observations are known to lie at or below a threshold, for example, a modelled median above that threshold cannot be reconciled with the censoring information. Suppressing such point estimates distinguishes model extrapolation from quantities identifiable within the data. Importantly, this gate affected descriptive quantiles and derived fold changes only; it did not modify Peto–Peto inference or FDR control.

Analytical provenance was equally important. Hair reference intervals show substantial inter-study and inter-laboratory variability, with sample preparation, digestion, instrumentation, population definition and analytical sensitivity recognised as major sources of heterogeneity [11,12]. Digestion protocol can also affect element recovery and reproducibility in ICP–MS analysis [13]. Historical ICP–OES and later ICP–MS measurements were therefore treated as complementary analytical datasets rather than interchangeable measurements, with one audited participant-level source assigned to each element and the same designated source used in both developmental strata.

This was particularly consequential for Ni and V. Both were present in the historical child ICP–OES panel but not the published adolescent legacy panel; combining legacy child results with later adolescent ICP–MS values would have confounded developmental stage with analytical platform. Ni and V were therefore derived from the later ICP–MS campaign in both strata. Likewise, legacy variables whose historical processing could not be reconstructed sufficiently were gated rather than completed through undocumented assumptions.

The same principle motivated the observed-only STRICT CORE. PCA and PERMANOVA were restricted to seven completely observed, positive and uncensored elements in all 217 participants, while wider CORE and EXPANDED panels were retained as assumption-dependent sensitivities. Their broadly concordant distance structures support the interpretation that the main multivariate result was not an artefact of selecting only seven variables. This hierarchy—fully observed primary inference followed by explicitly model-dependent sensitivities—provides a reproducible strategy for heterogeneous multielement biomonitoring datasets.

4.6. Robustness of the Principal Conclusions

The stability of the developmental fingerprint across prespecified analytical decisions strengthens the principal interpretation. Reconstructing the historical $N = 117$ child and $N = 96$ adolescent populations preserved all developmental FDR classifications and essentially all comparable effect directions, with near-perfect concordance of numerical effects. The findings therefore do not depend on retaining the small number of participants excluded from earlier publications.

Alternative analytical-limit definitions produced similarly reassuring results. Both reconstructed procedural-blank 3σ limits and the separate documentary instrument-file threshold preserved all comparable developmental FDR classifications, with strongly correlated effect estimates. The platform-restricted analysis using later ICP–MS variables alone likewise showed complete concordance among the directly comparable point-estimable effects. These findings do not imply that LoD definition or analytical platform is inconsequential—individual estimates changed and heavily censored elements remained uncertain—but they indicate that the principal developmental pattern is not generated by one particular limit definition or by inclusion of legacy ICP–OES variables.

Method sensitivity captured a different dimension of uncertainty. Ag, Co, Rb and V differed in FDR classification between the prespecified primary and permutation procedures and were therefore labelled method-sensitive rather than reclassified according to whichever method yielded the smaller *p*-value. Findings reproduced across population, analytical-limit, platform and methodological sensitivities should consequently be regarded as more robust than associations dependent on a single inferential specification.

4.7. Sparse and Context-Dependent Element Co-Variation

The conservative network analysis retained only four of the 21 possible STRICT CORE pairs. Fe–Cu was positive in both children and adolescents, Fe–Mo was additionally positive in children and Zn–Mo was negative in children. Replication of Fe–Cu across both developmental strata is noteworthy, but these remain statistical co-variations and cannot establish a shared exposure source or biological pathway.

Human-hair elements can form correlated groups, including associations among alkaline-earth elements [60], and correlation structures may differ by sex [25]. Contemporary multivariate studies likewise suggest context-dependent elemental modules rather than a universal hair-element network [3]. Against this background, the sparsity of the present network is informative: FDR control, a minimum effect-size criterion and bootstrap stability removed most associations that would have appeared in an unrestricted correlation matrix.

The retained edges should therefore be considered a small set of reproducible within-cohort associations rather than an inferred biochemical system. Independent replication using harmonised contemporary paediatric datasets is required before generalisation. Future network analyses incorporating environmental and dietary matrices could help determine whether stable hair–hair associations reflect shared external sources, biological co-regulation or matrix-specific incorporation.

4.8. Strengths and Limitations

Several features strengthen this study. It integrates participant-level data from two well-defined paediatric developmental strata across an unusually broad elemental programme. Historical and later source files were cross-audited before integration; analytical provenance, censoring limits and censoring status were preserved; deterministic substitution was avoided in primary inference; and the principal multivariate analysis used completely observed data. Multiplicity control, PERMDISP, bootstrap stability, historical-population reconstruction, alternative analytical limits, platform-restricted analysis and broader multivariate panels

were incorporated into a frozen scripted workflow rather than selected after inspection of the final results.

The principal limitation is the historical and geographically restricted cohort. All specimens were collected in one municipality in 2001, so the data should not be interpreted as contemporary exposure estimates or reference values for the broader Madrid or Spanish paediatric population. Nevertheless, the historical sampling period is central to the scientific purpose of this investigation. The archived specimens provide a well-defined population-level baseline from an earlier period of environmental and technological exposure, allowing the examination of multielement associations within the original cohort and establishing a potential reference point for future temporal comparisons. Recent investigations of Ba–Sr–V, nutritionally relevant and mixed-source elements, and rare earth and technology-related elements using the same archived cohort have similarly demonstrated the analytical feasibility and scientific value of this historical material, while acknowledging the limitations associated with retrospective interpretation and long-term storage [39–41]. Importantly, these previous investigations do not constitute independent validation of the present integrated multielement findings, as they were based on the same participants. An independently recruited paediatric cohort, ideally using comparable age groups, residential classifications and harmonised analytical protocols, would be required to establish external reproducibility and evaluate potential temporal changes in elemental profiles.

The unequal sex distribution and relatively small subgroup sizes represent additional limitations. In particular, the adolescent group included only 29 males compared with 68 females, potentially reducing the statistical power and precision of sex-stratified comparisons, especially for elements with substantial left-censoring. Residential-zone analyses were also constrained by small adolescent subgroup sizes, particularly in Zone IV ($n = 17$) and Zone II ($n = 20$). Although non-parametric and permutation-based procedures were employed, these approaches do not eliminate uncertainty arising from limited or unbalanced subgroup sizes. Furthermore, the significant PERMDISP result in the adolescent residential analysis ($p = 0.0264$) indicates differences in within-zone multivariate dispersion, meaning that the corresponding PERMANOVA association cannot be attributed exclusively to differences in group centroids. These findings should therefore be interpreted cautiously and independently validated in larger, more balanced paediatric cohorts.

Archival storage introduces additional uncertainty. Hair is a durable keratinised matrix, but the study cannot establish whether approximately two decades of dry storage affected recoverability of every measured element. Long-term invariance has not been demonstrated for all elements and analytical conditions represented here. Direct comparison of absolute concentrations with newly collected contemporary hair should therefore be cautious unless storage effects are evaluated experimentally. Dedicated stability studies involving repeated analysis of aliquots or controlled storage experiments would be informative.

Hair itself is an element-specific biomarker. Biomarker validity and effective exposure windows differ across metals and metalloids [7]; external deposition may not be completely removed by washing [6,10]; and correspondence with blood or urine is inconsistent across elements [8,9]. The present measurements consequently cannot be translated directly into nutritional sufficiency, systemic body burden, toxicity or individual health risk. Restricting recruitment to naturally dark, untreated hair improved within-cohort comparability but limits generalisability to populations with different pigmentation, morphology or cosmetic practices.

Analytical heterogeneity is another unavoidable consequence of the retrospective design. Although the same standardised hair decontamination procedure was employed across analytical campaigns, historical ICP–OES and subsequent ICP–MS determinations differed in instrumentation, analytical sensitivity, and the timing of sample digestion and

analysis. The campaigns followed a common nitric acid digestion approach, although some operational conditions, including pre-oxidation duration, varied within the documented procedures. Furthermore, the historical elemental panels were not identical between developmental strata. Such variability is well documented in hair analysis [11–13]. Element-specific analytical provenance and the use of a common designated source within each comparison minimise potential confounding but cannot establish complete analytical equivalence between campaigns.

Finally, some historical information could not be reconstructed at the participant level. Al and Pb retained raw analytical information, but their historical processing of detection-limit observations and selected extreme values could not be reproduced with sufficient certainty; Mn contained ambiguous censoring/zero coding; and adolescent participant-level Be and Tl were unavailable. These variables were gated rather than reconstructed through undocumented assumptions. Participant-level diet, socioeconomic circumstances, activity, environmental measurements, hair-growth rates and pubertal stage were also unavailable. Residual confounding is therefore unavoidable, and developmental, sex and residential findings should be interpreted as structured population associations rather than causal pathways.

4.9. Implications and Priorities for Future Paediatric Hair Biomonitoring

Future paediatric hair biomonitoring should explicitly model developmental structure rather than treat age only as a nuisance covariate. Longitudinal sampling across pubertal transition, incorporating Tanner stage, growth measures and, where feasible, endocrine markers, could distinguish maturation from concurrent dietary, behavioural and environmental change.

Biological interpretation should be established element by element using contemporaneous blood, urine and, where useful, other matrices, particularly for emerging and technology-critical elements with limited biomarker validation [7,61]. Hair growth, morphology, pigmentation, cosmetic practices, diet and supplement use should also be recorded.

Spatial studies should pair hair with participant- or household-level soil, dust, drinking-water and dietary measurements, potentially supplemented by geochemical or isotopic tracers [32,33]. Prospective longitudinal or geographical comparisons should use harmonised analytical platforms, rigorous blanks, suitable reference materials and transparent LoD/LoQ procedures, especially for highly censored ultra-trace elements. Storage-stability experiments are likewise needed to define defensible retrospective comparisons.

Finally, the 2001 Alcalá cohort is best viewed as a historical biomonitoring baseline rather than a contemporary exposure profile. A new school-based survey using comparable age strata and geographical coverage and a single modern workflow would be particularly informative. Where sufficient archived material remains, analysing archived and contemporary specimens within the same batch could help separate temporal change from platform effects; independent cohorts are also needed to test generalisability. Recent independent research in a different paediatric population has also combined multielement scalp-hair measurements with PCA, PERMANOVA and false-discovery-rate correction, illustrating the applicability of multivariate biomonitoring approaches while highlighting the importance of population-specific interpretation [62].

5. Conclusions

This censoring-aware integration of a 42-element paediatric scalp-hair biomonitoring programme identified a reproducible multielement structure associated primarily with developmental grouping. In the fully observed seven-element STRICT CORE, the child–adolescent contrast accounted for approximately 10.8% of multielement variation

without evidence that the result was driven by unequal multivariate dispersion. Sex contributed smaller but reproducible associations within both developmental strata, while residential setting was associated with additional heterogeneity; the adolescent residential result required qualified interpretation because multivariate dispersion also differed among zones.

Element-specific developmental differences were widespread, but variable detectability constrained numerical effect estimation for several elements. Preservation of censoring information, element-level analytical provenance, an observed-only primary multivariate core and prespecified sensitivity analyses prevented analytical completion decisions from being treated as measured biological information. The high stability of the principal developmental findings across historical population and analytical-limit scenarios supports the robustness of the overall multielement pattern.

Scalp hair can therefore provide a useful population-level matrix for identifying structured multielement patterns when analytical and interpretative limitations are addressed explicitly. It should not, however, be treated as a universal surrogate for internal dose, nutritional status or health risk. For toxicological human biomonitoring, the principal methodological implication is that analytical detectability, provenance and matrix-specific limitations must be explicitly separated from biological or exposure-related interpretation. Prospective longitudinal, multi-matrix and pathway-resolved studies using harmonised analytical protocols are now required to determine the biological and environmental processes underlying the developmental, sex-associated and spatial patterns identified here.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/toxics14110988/s1: Supplementary Methods: SM1. Legacy ICP–OES analytical procedures and QA/QC; SM2. Later ICP–MS analytical campaigns and QA/QC; SM3. Analytical limits and analytical-limit sensitivity documentation; SM4. Historical population and participant-level data reconciliation. Supplementary Tables: Table S1. Analytical provenance, QA/QC, censoring, historical reconciliation and inferential eligibility; Table S2. Complete censor-aware inferential results for developmental stage, sex and residential zone, with adjusted developmental models; Table S3. Sensitivity, stability and validation analyses. Supplementary Figures: Figure S1. Robustness of developmental-stage effect estimates across historical population restrictions, a later ICP–MS-only analysis and alternative analytical-limit scenarios. Primary and sensitivity $\log_2$(adolescent/child) effect estimates are plotted against the line of identity; the panel annotation reports Spearman ρ. Elements lacking censor-compatible numerical point estimates are excluded from effect-size concordance but retain their primary inferential classification where applicable. Method-sensitive findings are identified separately; Figure S2. Robust element-association networks in children and adolescents using observed-only STRICT CORE concentrations. Edges are retained only when BH $q \leq 0.05$, |Kendall $\tau| \geq 0.25$, bootstrap sign stability is ≥80%, and the 95% bootstrap interval excludes zero. Solid lines denote positive associations and dashed lines negative associations; line width reflects association strength/stability. No network communities are inferred.

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Institutional Review Board Statement: The original study was conducted in accordance with the principles of the Declaration of Helsinki. The retrospective analytical study and reporting framework were approved by the University of Alcalá Research Ethics Committee (Comité de Ética de la Investigación y de Experimentación Animal, CEI-EA; protocol: CEIP/2025/3/089; approval date: 5 May 2025).

Informed Consent Statement: Written informed consent for participation in the original 2001 study was obtained from parents or legal guardians, with assent from participating minors where appropriate. No identifiable individual participant information is presented in this manuscript.

Data Availability Statement: De-identified derived analytical outputs, analytical metadata and materials documenting the reproducible statistical workflow are available in Figshare (DOI: 10.6084/m9.figshare.33407197). The reproducible R (R version 4.4.2) analysis code, including analysis scripts, the data dictionary, decision documentation, package and session information, fixed random seeds, and integrity manifests associated with the frozen analytical workflow, is available with the study materials in the same Figshare repository. The complete underlying participant-level dataset cannot be made publicly available because it contains historical data collected from minors under a consent framework that did not anticipate unrestricted open sharing of individual-level data. Requests for additional de-identified participant-level material may be directed to the corresponding author and will be considered subject to applicable ethical, institutional and data-protection requirements.

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