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Rationale

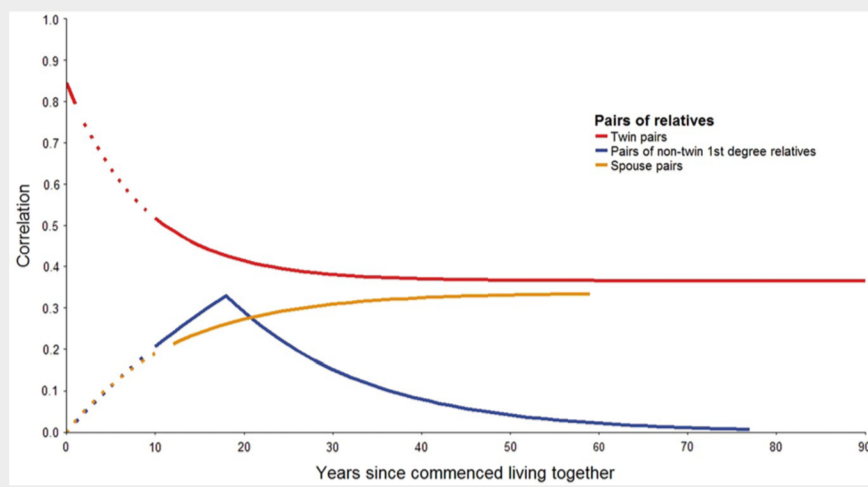


Figure 1. Correlation of methylation profiles stratified by cohabitation history from Li et al [1].

External exposome can shape the individuals' DNA methylation profile, especially in the crucial time window of exposures of the first 1000 days of life.

It has been shown that individuals sharing similar conditions within the same households have similar DNA methylation profiles (**Figure 1**, adapted from [1]).

We aimed to investigate whether these similarities were consistent also within individuals living in the same geographical area. The objective of this study is to investigate the association between geographical differences and differences in DNA methylation levels, first between different Italian cities and then locally within a single Italian city.

Methods



Figure 2. Geographical distribution of the PiccoliPiù DNA methylation samples across Italy.

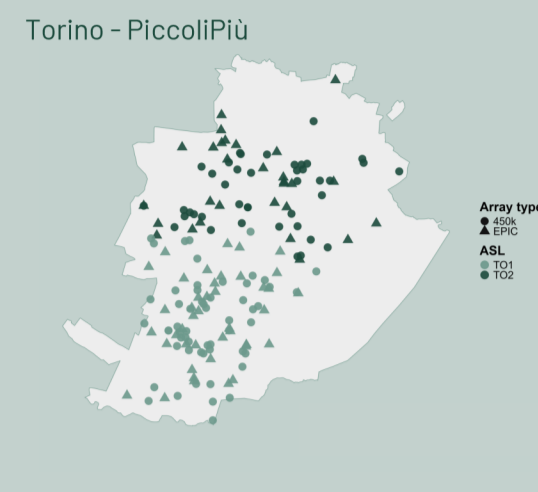


Figure 3. Geographical distribution of the PiccoliPiù DNA methylation samples across the city of Torino.

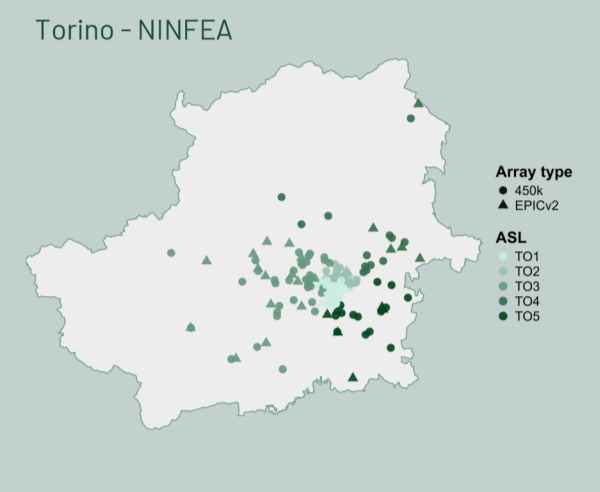


Figure 4. Geographical distribution of the PiccoliPiù DNA methylation samples across the province of Torino.

Between-cities analyses:

364 cord blood samples (PiccoliPiù birth cohort, **Figure 2**) from the birth centers in Roma, Firenze, Viareggio, Trieste and Torino.

- Differentially methylated probes (DMP) analysis to identify candidate CpGs between cities, stratified by DNA extraction buffer (collinear with the birth centers);
- Candidate CpGs: intersection of the top 500 DMP for each comparison stratified by buffer, appearing in at least three comparisons.

Within-city analyses, conducted in Turin:

169 cord blood samples (PiccoliPiù birth cohort, **Figure 4**), 189 saliva samples collected at 6-18 months (NINFEA birth cohort, **Figure 3**).

- DMP analyses between different local health service area (ASL);
- Geostatistical analyses analysing semivariance vs distance (variograms).

All analyses are adjusted for: sex, chip, and cell types. NINFEA analyses are also adjusted for age at sample collection.

Results

Between-cities analyses:

DNA from PiccoliPiù samples has been extracted into two different buffers, almost collinear with the city, thus it was impossible to disentangle the city effect from the buffer effect (**Table 1**). CpGs from the Firenze samples showed to be differentially methylated based on buffer of DNA extraction (**Figure 5**). We performed the between city comparisons to find candidate CpGs after stratifying for buffer of DNA extraction (**Figure 6**).

	DNA extraction buffer	
	ATE	H2O
Firenze	62	28
Roma	21	
Torino		72
Trieste	92	
Viareggio	89	

Table 1. Distribution of DNA extraction buffer based on the city. Cities are collinear with buffer except for Firenze samples.

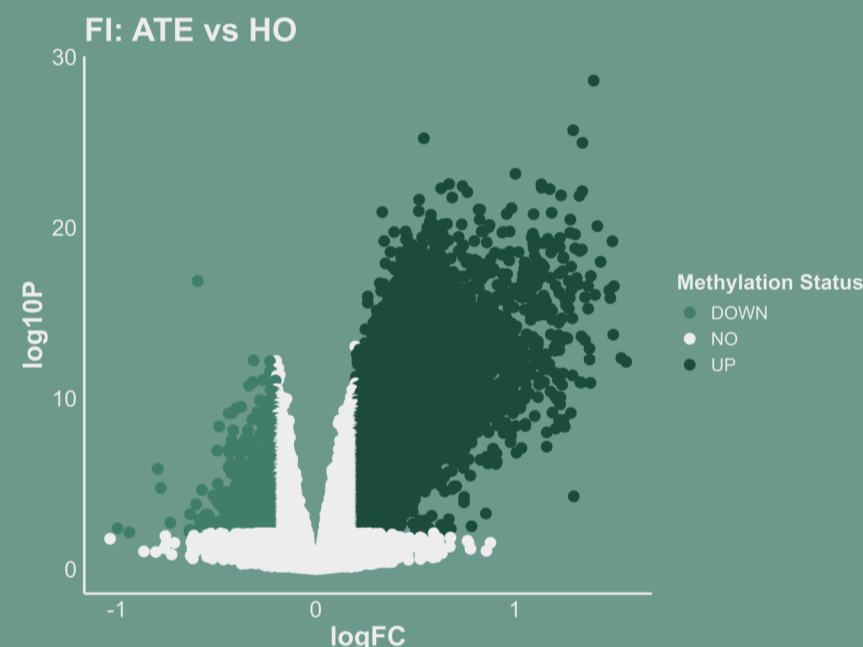


Figure 5. CpGs are differentially methylated by the DNA extraction buffer in Firenze samples.

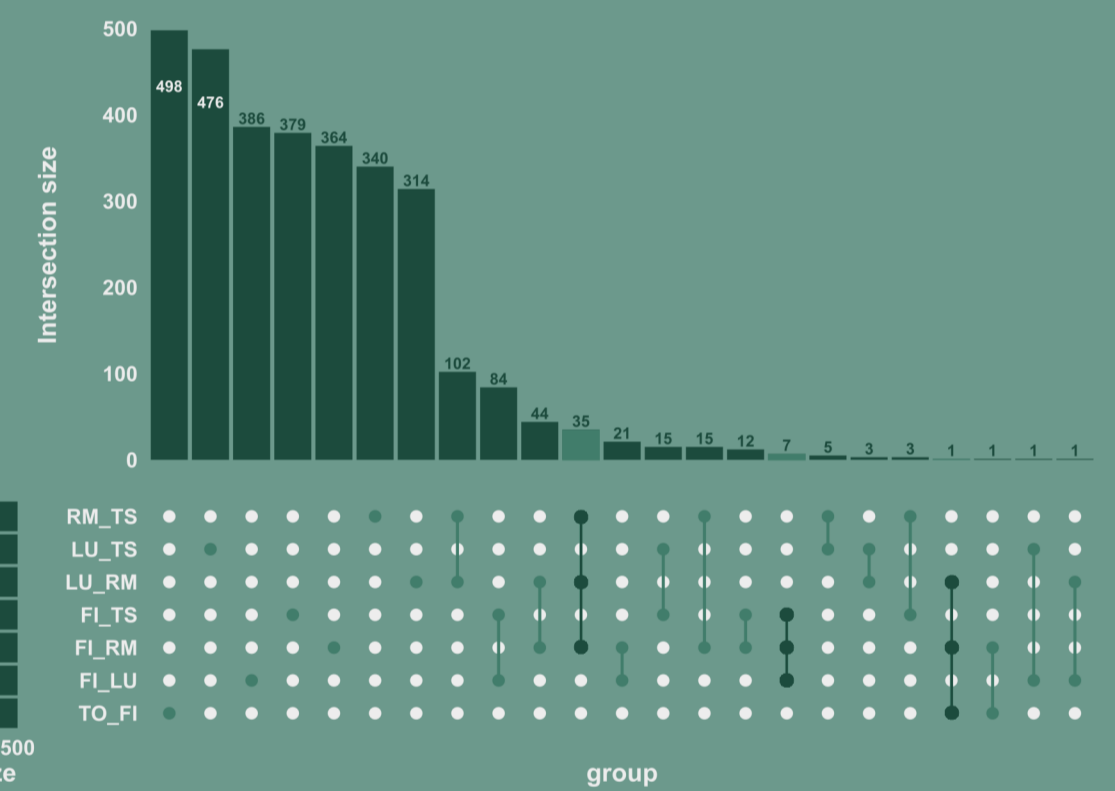


Figure 6. Intersection plot showing the intersection between the top 500 differentially methylated CpGs in every comparison. The 43 candidate CpGs are selected as the ones appearing in at least three comparisons (highlighted).

Within-city analyses:

No DMPs across different ASLs are found in the candidate CpGs from the previous analyses, nor in the NINFEA (**Figure 7**) nor in the PiccoliPiù (**Figure 9**) samples.

Variograms do not show evidence of an association between the methylation levels of CpGs and geographical distance between subjects, nor in NINFEA (**Figure 8**), nor in PiccoliPiù (**Figure 10**) samples.

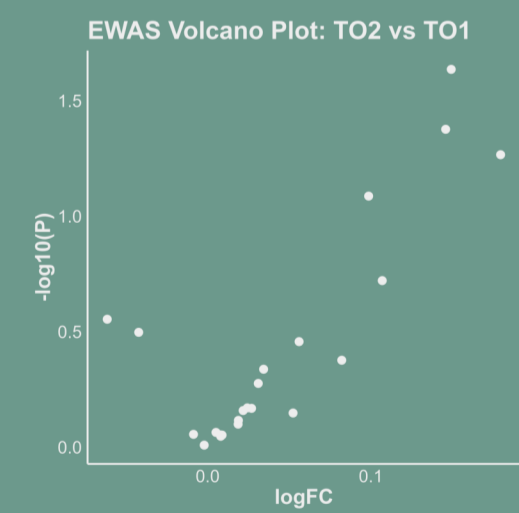


Figure 7. Volcano plot showing no DMPs by ASL in the Torino province (NINFEA cohort). Only results from the comparison between two ASLs is shown for simplicity.

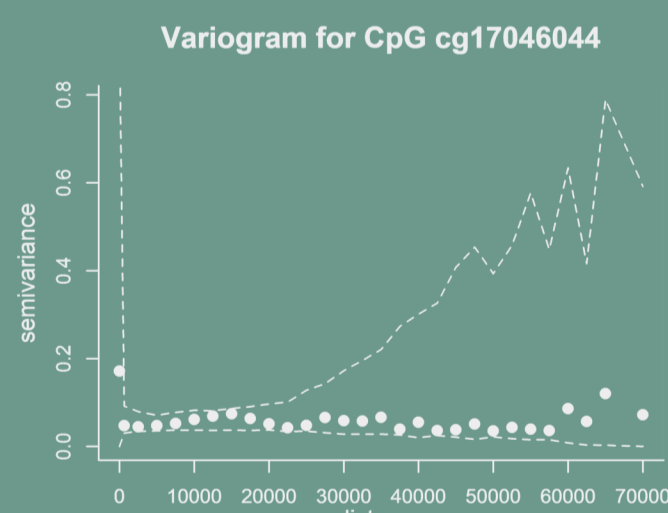


Figure 8. Variogram of the methylation level of one candidate CpG versus the distance between any couple of subjects (NINFEA cohort). Only results from one candidate CpG are shown for simplicity.

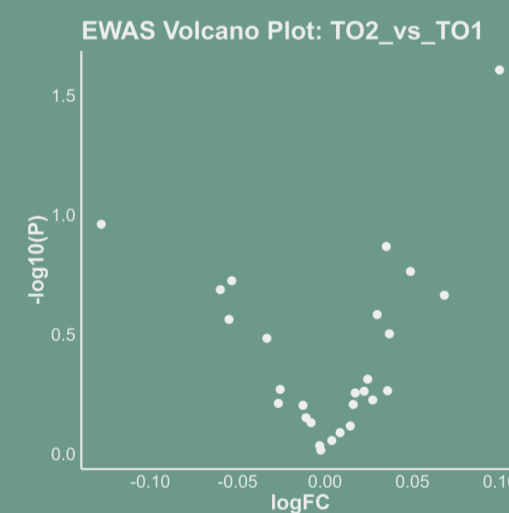


Figure 9. Volcano plot showing no DMPs by ASL in Torino (PiccoliPiù cohort).

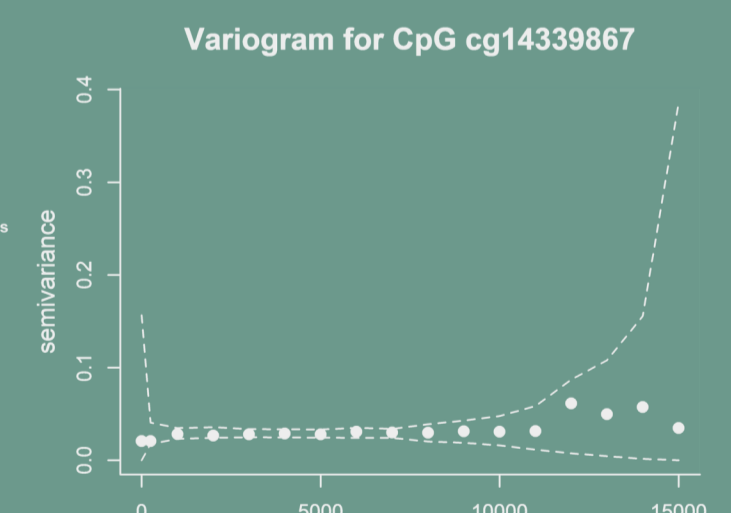


Figure 10. Variogram of the methylation level of one candidate CpG versus the distance between any couple of subjects (PiccoliPiù cohort). Only results from one candidate CpG are shown for simplicity.

Discussion and conclusions

- To our knowledge this study represents the first attempt of linking geographical distance with variation in DNA methylation profiles. Our results suggest that the geographical area of birth influences the methylome, reflecting a response to regional environmental stressors.
- In contrast, effects were limited within the same area of residence. This suggests that at a local level methylation differences might be driven by non-linear factors. Distant neighbors sometimes share similar exposures, masking distance-based trends within a single city.
- While the small sample size is a limit for this study, it serves as a proof-of-concept. Further research with larger and more diverse cohorts would help disentangle the relationship between geographical distance and DNA methylation profiles.

Funding

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References

- Li, S. et al. (2018) *Int J Epidemiol.* **47**(3):908-916.