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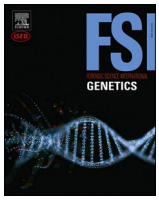

Research paper

Non-self DNA on the neck: a 24 hours time-course study

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ABSTRACT

Non-self DNA is normally present on skin due to DNA transfer occurring during daily activities. The understanding of persistence and accumulation of foreign DNA on the neck can assist in the interpretation of DNA evidence collected from an assaulted victim. Establishing the composition and level of non-self DNA present is relevant, especially in cases where the victim cohabits with other individuals, such as partner and children. This study investigated the persistence and accumulation of non-self DNA on the neck, over the course of 24 h. DNA samples were collected from the neck of 20 adult volunteers at three time-points, on two days. The detection of a partner's DNA and DNA from unknown sources was studied in relation to the living arrangement and to the activities performed by each individual. An increased number of non-self alleles were detected over time. Partner's DNA was observed to accumulate during the day and to persist when an individual was absent from the shared home environment. DNA from unknown contributors was found on the neck of individuals that used public transport, attended public spaces and had social interactions. The data acquired from this study will help to increase knowledge on the composition of DNA present on an individual's neck in a daily situation.

1. Introduction

During the investigation of physical assaults, samples may be collected from areas of the victim's skin where physical contact with the offender could have occurred [1,2]. The transfer of DNA and the possibility to collect offender's DNA from victims' skin after a physical assault has been demonstrated by several studies [1–5]. For example, Wiegand and Kleiber [1] and Rutty [3] investigated how DNA profiling could assist in the identification of the perpetrator of manual strangulation. In addition to the detection of suspect's DNA, the presence of "normal" non-self DNA on adult necks was demonstrated by Graham et al. [4]. Non-self DNA is typically deposited on the neck surface during innocent daily activities and is present at a level that can be detected with standard forensic methods [2,4]. A person's shedder status could influence the amount of DNA that he or she will deposit upon contact with items or surfaces, such as another person's skin [6–11]. When DNA is transferred to skin, the shedder status of the receiving individual could affect the amount and composition of DNA that is recovered [12,13]. The DNA shedding ability is just one of the factors that may influence the recovery of foreign DNA from skin. Co-habitation and relationship status were also observed to have an effect on the quantities of non-self DNA found on skin. High quantities could be present on the neck's surface of co-habiting individuals [2,4]. In many criminal cases the offender and the victim may cohabit or be in a relationship, thus DNA could be innocently transferred at a time unrelated to the crime-event [2]. In court, known previous contact(s) of a victim with a suspect is often used as a defense proposition when evaluating the evidence at activity level [14,15]. Knowledge on levels and composition of non-self DNA in normal conditions are important for the assessment of activity related questions in cases where samples have been collected from skin [4,16]. The evaluation of DNA evidence given propositions at activity level can be conducted using Bayesian networks. Bayesian networks are probabilistic graphical models that consist of nodes, arcs and probability assignments. The network uses Bayesian inference for probabilistic assessments. Data derived from controlled experiments can be used to inform the models for activity level interpretation of crime scene evidence [17,18]. The purpose of this study is to increase the understanding of persistence and accumulation of non-self DNA on the neck, over the course of 24 h, and to create a dataset that can aid in the evaluation of evidence at activity level. To do this, we investigated levels of non-self DNA on the neck over a period of time since washing. The detection and persistence of partner's DNA and DNA from unknown individuals was studied, along with the influence of the living arrangement and daily activities of a subject.

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2. Materials and methods

2.1. Ethical declaration

This study was approved by the data protection officer (DPO) at Oslo University Hospital (reference: 20/20155). All participants delivered informed consent before participation in the study.

2.2. Samples collection

Twenty adult volunteers (13 females and 7 males) were recruited for this study. Each individual was instructed to collect six DNA samples by swabbing specific areas of the neck surface. A sampling kit was delivered to each participant. The kit contained: detailed instructions on the sample collection procedure, an envelope with cotton swabs and sampling bags for reference samples (oral mucous) collection, a second envelope with pre-labeled sampling bags, cotton swabs and distilled water for sample collection. Each sampling bag was labeled with the participant identification code, the sampling area and the sampling time-point. Participants were asked to report the time and date of collection on each sampling bag. A total of 120 samples were collected and analyzed. The sampling was repeated on two different days: one weekday and one day over the weekend. This enabled detection of differences in non-self DNA levels on the two days: a workday, where the participant spent most of the time at the workplace and a weekend, where the participant stayed in his/her home environment. Participants were instructed to take a shower in the morning and to not wash the sampling area (neck) until all samples were collected. Each participant was asked to use a clean towel, not to apply any type of cosmetic product on the sampling area before collecting the first sample and to go about the day normally. The samples were collected by each participant using a moistened cotton swab at three different time-points, over the course of 24 h: 0 h after the shower (T_0), 7–8 h after the shower (T_1) and 24 h after the shower (T_2), (Table 1). On the weekday, participants were asked to collect T_1 sample when they were at their workplace. The samples were collected from three different neck areas: front, right and left and back of the neck respectively (Table 1, Fig. 1), since multiple swabbing on the same skin area would have affected DNA persistence. Each sample was collected by swabbing a large skin surface, as this maximized the amount of DNA that could be recovered [4]. All sample donors provided their reference DNA profiles for comparison with the resultant DNA profiles. Participants that lived together with their partner were also invited to provide a reference sample from their partner. Thirteen of the 16 participants in a relationship provided their partner's reference sample. This allowed study of persistence and accumulation of partner's DNA over time.

2.3. Control study

A control study was conducted to investigate whether at T_0 the three neck areas displayed a different DNA-quantity. Three volunteers collected three samples from the different neck areas (Fig. 1) at T_0, just after a shower. The samples were processed and analyzed following the same procedures adopted in the main study.

2.4. Activities questionnaire

Each participant was asked to fill in a questionnaire to discover: the relationship status, the living arrangement of the individual and the activities performed during the two sampling days and between the three sampling time-points. For each activity the participants recorded the time and day of when it was performed. This enabled an analysis of association of personal habits and activities with the DNA profile results. Details on the variables investigated in the questionnaire are provided in Supplement S1, Table S1.

Table 1
Time-points and sampling areas.

Time-points		Sampling areas
T_0:	0 h after the shower	front of the neck
T_1:	7–8 h after T_0	right and left side of the neck
T_2:	24 h after T_0 (before the shower)	back of the neck

2.5. Sample processing

All samples were processed in a "DNA-free" investigation room pre-cleaned with 0.1% hypochlorite solution. Personal protection equipment (lab coat, face mask, hair net and gloves) was worn by the individual processing the samples to avoid any contamination. Gloves were repeatedly changed and cleaned with 70% ethanol between each sample. Benches were cleaned with 0.1% hypochlorite solution and new bench paper was used when samples from a new participant were handled. Each cotton swab was extracted from its sampling bag; the cotton swab's tip was cut and placed into a 1.5 mL extraction tube. All samples were stored at room temperature until DNA extraction.

2.6. DNA analysis

DNA extraction was performed according to Chelex® 100 (Bio-Rad Laboratories) protocol, using 200 μl of 5% Chelex solution (also elution volume). No prior incubation with water was made [19,20]. Negative extraction controls were processed with the samples. The amount of DNA in each sample was determined using PowerQuant® System (Promega) on the 7500 RealTime PCR System (Applied Biosystems) following manufacturer's instructions. The extracted DNA was amplified using PowerPlex® Fusion 6C System (Promega). 1.0 ng of template DNA was used for PCR amplification in a total reaction volume of 25 μl. 29 PCR cycles were used for amplifying the samples on Applied Biosystem® Veriti 96-Well Thermal Cycler (ThermoFisher). Negative and positive amplification controls were run together with the samples. Amplification products were separated and detected by capillary electrophoresis on the Applied Biosystem® 3500xL Genetic Analyzer (ThermoFisher). An injection time of 24 s and a voltage of 1.2 kV were applied.

2.7. Data analysis

The data were analyzed on GeneMapper ID-X v.1.6 (Life Technologies) with an analytical threshold (AT) set to 100 RFU. The results were analyzed by two different researchers. Technical artefacts and stutters were filtered from the DNA profiles, according to internal guidelines. All profiles were compared with that of the respective donor and, when available, with the partner's reference profile. When mixtures were detected, the minimum number of contributors was calculated using the allele counting method described by Clayton et al. [21]. The number of alleles matching the donor, the number of unique partner's alleles along with alleles from unknown contributors were counted for each DNA profile. An allele from an unknown contributor was recorded provided that its peak height was greater than the AT and it was not present in the reference profile of the sample donor and partner. The total number of alleles was obtained by the sum of donor's alleles, unique partner's alleles and unknown contributors' alleles. The number of non-self alleles was defined as the sum of unique partner's alleles and alleles from unknown individuals. Amelogenin and Y-STR alleles were not considered in the calculation of donor and unique partner's alleles, whereas Y-STR alleles were considered when from unknown individuals. Homozygous alleles were counted as two where a reference profile was available for comparison. When alleles from unknown contributors and alleles from a partner that did not provide the reference sample were detected, both heterozygotes and homozygous alleles were counted as one.

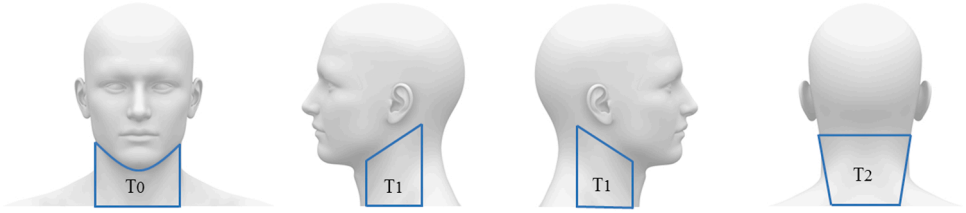

Fig. 1. Sampling areas: T_0: front of the neck; T_1: right and left side of the neck. T_2: back of the neck.

2.7.1. EuroForMix analysis

The DNA profiles were exported from Genemapper ID-X v.1.6 (Life Technologies) for a secondary analysis on EuroForMix v.3.2.0 (EFM) [22]. An automated analysis was carried out with an R-script macro that used EuroForMix to calculate the average RFU, mixture proportions (M_x) if mixtures were present and the likelihood ratio (*LR*) for the samples from individuals living with partner (and children) for whom the partner's reference sample was available. The analysis in EFM was run without the stutter model, as stutters were previously filtered from the profiles; the degradation model was used. Fixation index (*Fst*), drop-in probability (P_c) and λ parameters were set as follows: $Fst = 0.01$, $P_c = 0.05$, $\lambda = 0.01$. The prosecution (H_p) and defense (H_d) propositions for *LR* calculations were set as described by Eqs. (1)–(4). The numerator was conditioned upon the *POI* and the victim (*V*) whilst the denominator was conditioned on the victim only. The sample donor was considered as victim and the partner as *POI*. Unknown contributors are reported as "U_x". LR_{rel} values were calculated to establish whether alleles from a child of the *POI* (U_{child}) were present in the DNA profile, rather than alleles from the *POI*.

$$LR = \frac{\Pr(E|H_p : POI + V)}{\Pr(E|H_d : V + U_1)} \quad (1)$$

Two-person mixture

$$LR_{rel} = \frac{\Pr(E|H_p : POI + V)}{\Pr(E|H_d : V + U_{child})} \quad (2)$$

Two-person mixture considering relatedness (child)

$$LR = \frac{\Pr(E|H_p : POI + V + U_1)}{\Pr(E|H_d : V + U_1 + U_2)} \quad (3)$$

Three-person mixture

$$LR_{rel} = \frac{\Pr(E|H_p : POI + V + U_1)}{\Pr(E|H_d : V + U_1 + U_{child})} \quad (4)$$

Three-person mixture considering relatedness (child).

2.7.2. Adjusted average RFU

The average RFU was calculated for each DNA profile by summing the peak height values of all detected alleles (RFU_{tot}) and dividing the result by the total number of loci (*n*); Amelogenin and Y-STR markers were excluded from the calculation. The average RFU was adjusted for the samples that were diluted prior to amplification. The RFU values were multiplied by the dilution factor (d_l) of the diluted samples as described by Gill et al. [23].

The adjusted average RFU was further corrected by multiplying the mixture proportion of the sample donor (M_{X1}) for the evaluation of interpersonal differences.

$$RFU = \frac{RFU_{tot}}{n} \times d_l \quad (5)$$

2.8. Statistical analysis

ANOVA, t-test, Wilcoxon and Kruskal–Wallis statistical tests were carried out to evaluate if the expected values were the same in different groups at a 5% ($p < 0.05$) significance level. R v.4.1.1 (www.r-project.org) was used to perform the statistical analyses and the package ggplot2 v.3.3.3 to generate the plots displayed in this manuscript. For all boxplots presented in this study the upper whisker extends from the third quartile (Q3) to the largest observation and no further than 1.5 × IQR from Q3, where "IQR" is the inter-quartile range. The lower whisker extends from the first quartile (Q1) to the smallest observation, at most 1.5 × IQR of Q1. Data outside the whiskers range are defined as outliers and are plotted individually as black dots.

3. Results

3.1. Participants and samples overview

Twenty adult volunteers participated in this study. Each participant collected six DNA samples from the neck surface on established days and various time-points. The exact sampling date and time was recorded in each pre-labeled sampling bag. A total of 120 DNA samples were collected and processed. Two samples belonging to T_0 time-point were later excluded from the dataset, since they had been collected some hours after the participant took a shower. All the participants completed the activities questionnaire. Four of the participants declared to be single at the moment of the experiment, six declared to have a partner and ten declared to have a partner and children. Of the 16 individuals that stated to have a partner, 13 provided their partner's reference sample, whilst three declined. Overall, the sample donors declared to cohabit with 0–5 other persons: alone (n = 2), partner (n = 4), partner and children (n = 10), flatmates (n = 2) and partner and flatmates (n = 2).

3.2. Control study

All the samples from the control study resulted in single donor profiles. DNA concentration values varied between individuals and neck areas. Two of the participants displayed the highest amount of DNA in the sample collected from the sides of the neck while the third participant showed the highest amount of DNA in the sample collected from the front of the neck. On average, the lowest DNA concentration and adjusted average RFU values were displayed from the samples collected from the back of the neck that resulted in partial donor profiles. Further details on the control study results are provided in Supplement S2, Table S2.

3.3. DNA-quantity and alleles count

The DNA concentration ranged from a minimum of 4×10^{-4} ng/μl to a maximum of 4.69 ng/μl, with a mean value of 0.18 ng/μl. Inter- and intra-person variations in the DNA-quantity were observed. From the samples with low DNA concentration values, one sample displayed zero alleles, whilst 17 resulted in partial donor profiles, with or without foreign alleles. Overall, 44 single donor profiles and 73 mixtures of two to three contributors were detected. The positive and negative amplification controls resulted as expected. The total number of alleles detected ranged from a minimum of 0 to a maximum of 84 (mean = 53). The

number of donor's alleles ranged from 0 to 46 (mean = 44), with 46 alleles corresponding to a full DNA profile. The number of non-self alleles ranged from 0 to 38 (mean = 9). The number of unique partner's alleles detected ranged from 0 to 34 (mean = 9). The number of unknown alleles ranged from 0 to 12 (mean = 0.5). For details about the alleles detected for each sample we refer to Supplement S3, Table S3.

3.4. Composition of DNA profiles

Full and partial donor profiles were obtained from 38% of the samples, whilst 53% of the samples resulted in two-person mixtures and 9% in three-person mixtures. All two-person mixtures were donor-partner mixtures (where the partner's reference profile was available) except for one mixture from a single individual that displayed alleles from an unknown contributor. The three-person mixtures comprised donor, partner and an unknown contributor. The sample donor was the major contributor in all but three DNA mixtures. Two of these mixtures were from participants that provided the partner's reference sample and that declared on the questionnaire to have spent time with the partner and have had physical contact with him/her before collecting the samples. The third mixture was from a participant living with partner and children, who did not provide any additional information and for whom the partner's reference sample was not available for comparison.

3.5. Interpersonal differences

The adjusted average RFU (Eq. (5)) corrected by M_x (donor contribution) was applied to the 20 sample donors to evaluate differences between samples from different individuals (Fig. 2). Two individuals (C1 and V1) had the lowest donor contribution. Respectively, three and four samples from individuals C1 and V1 resulted in partial donor profiles and one sample from the first individual displayed zero alleles (Supplement S3, Table S3). At the other extreme, individuals F1 and E1 had the greatest donor contribution. The difference in the adjusted average RFU values (of sample donor) between the two individuals displaying the lowest donor contribution and the two individuals displaying the highest donor contribution was found to be statistically significant (Wilcoxon, $p < 0.05$). To assess how the differences observed in donor contributions influenced detection of foreign DNA, the number of non-self alleles detected was compared between individuals (Supplement S4, Fig. S4). An interesting result was displayed from participant F1 that showed a high sample contribution and a low number of non-self alleles. Although this individual declared to be living with the partner, only one sample from this subject displayed (two) partner's alleles. This individual could potentially be a high shedder. Participant E1 also displayed a high donor contribution and samples from this individual did not display any non-self alleles. This individual declared to be living alone. The two individuals that displayed the lowest donor contribution did not display a higher number of non-self alleles compared to the average. The number of non-self alleles detected at the three time-points was also compared between male and female donors (Supplement S4, Fig. S4.1). An increased number of non-self alleles were observed for males in contrast to females, however this was not statistically significant.

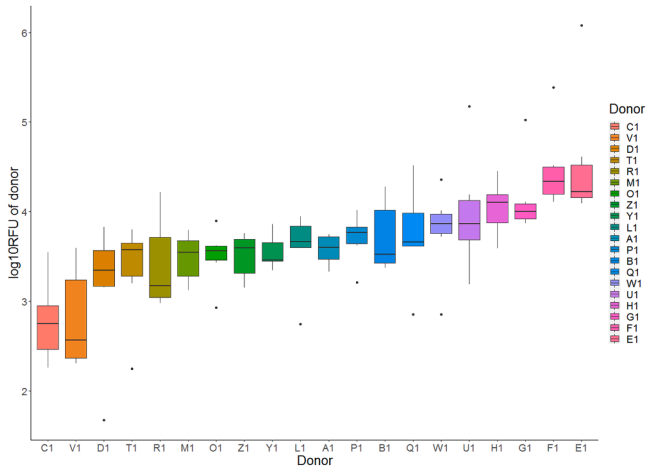

Fig. 2. Box plots displaying the $\log_{10}$ of the adjusted average RFU (log10RFU) of sample donors.

3.6. DNA concentration

The DNA concentration of all samples was analyzed over the three sampling time-points and the two sampling days. No significant difference was observed in the DNA-quantity between the three sampling time-points (ANOVA, $p = 0.3$) and between the samples collected during the weekday and the samples collected on the weekend (t-test, $p = 0.8$). The DNA concentration values for each sample are displayed in Supplement S3, Table S3.

3.7. Adjusted average RFU

The adjusted average RFU of all samples was investigated over the three sampling time-points and the two sampling days. No significant difference was identified for the RFU values between the three time-points (Kruskal–Wallis, $p = 0.09$) and between the two sampling days (Wilcoxon, $p = 0.5$). The adjusted average RFU values for each sample are reported in Supplement S3, Table S3.

3.8. DNA mixtures

The number of DNA mixtures detected increased over the three time-points. At T_0, 58% of the samples resulted in single donor profiles while 42% resulted in two-person mixtures. Thirty percent of the samples collected at T_1 yielded a single donor profile, 57.5% resulted in two-person mixtures and 12.5% in three-person mixtures. From the samples collected at T_2, 26% resulted in single donor profiles, 59% resulted in two-person mixtures and 15% in three-person mixtures (Fig. 3A). The differences in the number of mixture contributors at the three time-points were found to be statistically significant (Kruskal–Wallis, $p < 0.05$). Considering all time-points, on the two different sampling days 44% of the weekday samples resulted in single donor profiles, another 44% resulted in two-person mixtures and 12% in three-person mixtures. A higher number of two-person mixtures were detected from the samples collected on the weekend, where 31.7% of the samples resulted in single donor profiles, 61.7% in two person mixtures and 6.7% resulted in three-person mixtures (Fig. 3B). The difference in the number of mixture contributors between the two sampling days was however not statistically significant (Wilcoxon, $p = 0.4$).

3.9. Sample contribution

Analysis of mixture proportions (M_x) enabled the evaluation of the sample contribution from the sample donor (C1), the partner/second contributor (C2) and an unknown contributor (C3). C2 includes unknown contributors assumed to be partners who have not been tested (based upon prevalence of their profile in multiple samples tested). Significant differences in the sample contribution from the sample donor (C1) were found over the three sampling time-points, with a decrease in the M_x value over time from T_0 to T_2 (Kruskal–Wallis, $p < 0.05$). As the contribution from the sample donor (C1) decreased, the contribution from the partner/second contributor (C2) increased (Kruskal–Wallis, $p < 0.05$). Unknown contributors were detected only at T_1 and T_2 with higher contribution for samples collected at T_1, however, this result was not statistically significant (Kruskal–Wallis, $p = 0.2$), (Fig. 4). No significant difference was found for C1, C2 and C3 contributions between the two sampling days.

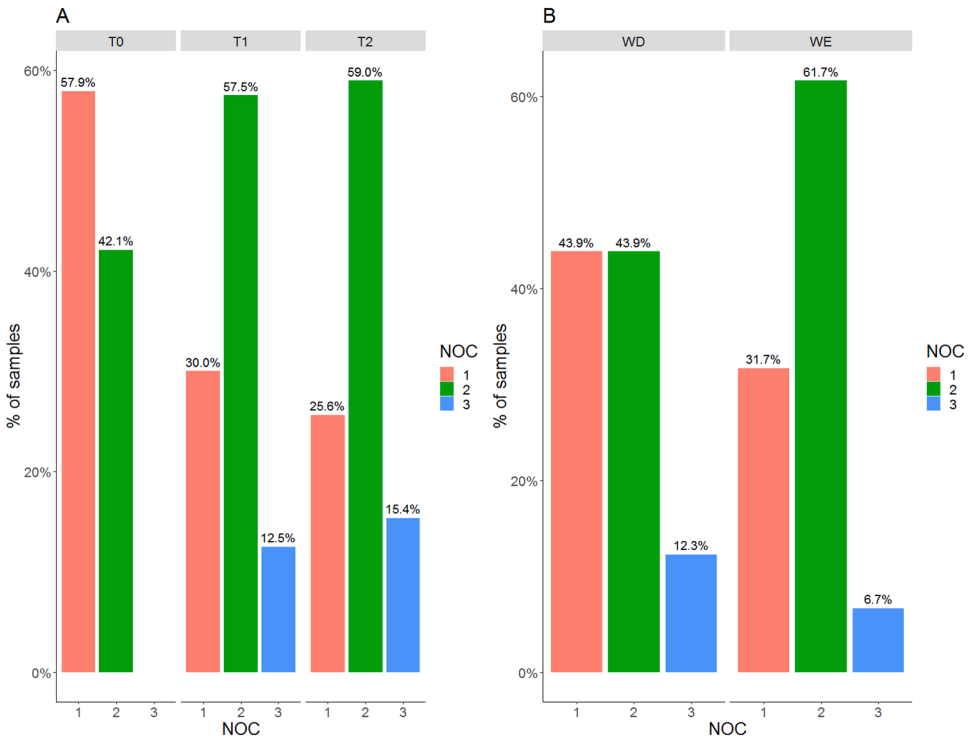

Fig. 3. A. Percentage of DNA mixtures for the three different time-points ($T_0 = 0$ h after the shower, $T_1 = 7$–8 h after the shower and $T_2 = 24$ h after the shower). The samples collected during the weekday and weekend are combined in the plot. B. Percentage of DNA mixtures for the two sampling days (WD = weekday, WE = weekend). The samples from the three time-points are combined in the plot. NOC = number of contributors.

3.10. Non-self DNA

The number of non-self alleles was investigated over the three time-points and the two sampling days (Fig. 5). A significant increase in the number of non-self alleles detected over time was found (Kruskal–Wallis, $p < 0.05$), with the highest number of alleles detected for the samples collected at T_2. More non-self alleles were detected for the samples collected during the weekend (WE), than for the samples collected in the weekday (WD); however the difference in the number of alleles detected between the two days was not significant (Wilcoxon, $p = 0.2$).

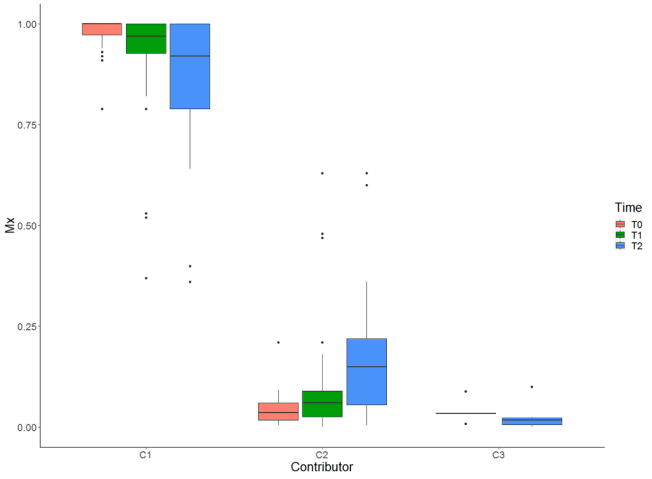

Fig. 4. Sample contribution over the three sampling time-points ($T_0 = 0$ h after the shower, $T_1 = 7$–8 h after the shower and $T_2 = 24$ h after the shower) for sample donor (C1), partner/second contributor (C2) and an unknown contributor (C3). M_x = mixture proportion.

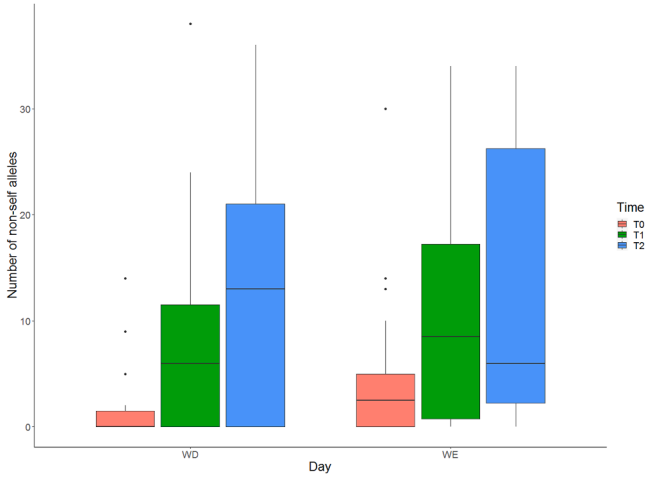

Fig. 5. Box plots displaying the number of non-self alleles detected vs the three sampling time-points ($T_0 = 0$ h after the shower, $T_1 = 7$–8 h after the shower and $T_2 = 24$ h after the shower) and the two sampling days (WD = weekday, WE = weekend).

3.10.1. Number of non-self alleles for different living arrangements

The number of non-self alleles detected was analyzed considering the different living arrangements of sample donors (Fig. 6). Significant differences were observed in the number of non-self alleles detected for different living arrangements (Kruskal–Wallis, $p < 0.05$). Pairwise comparisons (Wilcoxon) were conducted to evaluate the differences between combinations of living arrangements; the number of non-self alleles was significantly different ($p < 0.05$) for all combinations except for the combinations "flatmates vs alone" ($p = 0.4$) and "partner vs partner and children" ($p = 0.09$).

Participants living alone (n = 2) and participants sharing the house

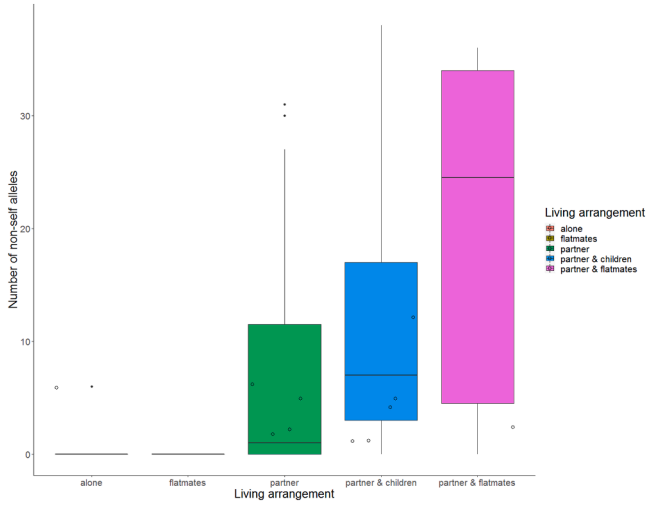

Fig. 6. Box plots displaying the number of non-self alleles detected vs the living arrangements of sample donors: living alone ("alone", 12 samples), living with flatmates ("flatmates", 12 samples), living with a partner ("partner", 23 samples), living with partner and children ("partner & children", 59 samples), living with partner and flatmates ("partner & flatmates", 12 samples). The white dots represent the number of unknown alleles detected.

with flatmates (n = 2) showed a similar result, with all the samples displaying single donor profiles, excluding one sample of a participant living alone that displayed six additional unknown alleles. More alleles were detected in the samples of participants cohabiting with the partner (n = 4), with the number of non-self alleles ranging from a minimum of 0 to a maximum of 31 (mean = 8). A higher number of alleles was detected in the samples from participants sharing the house with partner and children (n = 10), with the total number of alleles ranging from 0 to 38 (mean = 10). On average, the highest number of non-self alleles was detected in the samples from participants sharing the house with both partner and flatmates (n = 2), with the total number of alleles ranging from 0 to 36 (mean = 21). However, only one sample from the last group of participants displayed unknown alleles.

3.11. Partner and children's DNA

No children reference DNA samples were collected for this study for reasons of privacy. LR_{rel} values were calculated (Eqs. (2) and (4)) to investigate the proposition that alleles from a child of the *POI* (U_{child}) were present in a DNA profile. One sample from a participant with children gave an LR_{rel} of 1.3×10^{-6} (Eq. (4)) (i.e much lower than 1); the evidence supports the proposition that alleles from an untyped child were present rather than the alternative that alleles from the *POI* (partner) were present. For this sample, the presence of child's alleles was inferred by manually evaluating the DNA profile. This sample was then not considered for the count of unique partner's alleles.

3.11.1. Percentage of unique partner's alleles

The percentage of unique partner's alleles was calculated for the samples where the partner's reference profile was available (n = 76 samples). A significant increase in the percentage of unique partner's alleles was observed over the three time-points (Fig. 7), with the lowest percentage values for samples collected at T_0 and the highest for samples collected at T_2 (Kruskal–Wallis, $p < 0.05$). A difference in the percentage of partner's alleles detected was observed between the two sampling days, with more alleles detected from the samples collected during the weekend than from the ones collected during the weekday, however this was found to be not statistically significant (Wilcoxon, p = 0.2). Five of the samples collected during the weekend and one of the samples collected during the weekday displayed 100% of unique partner's alleles

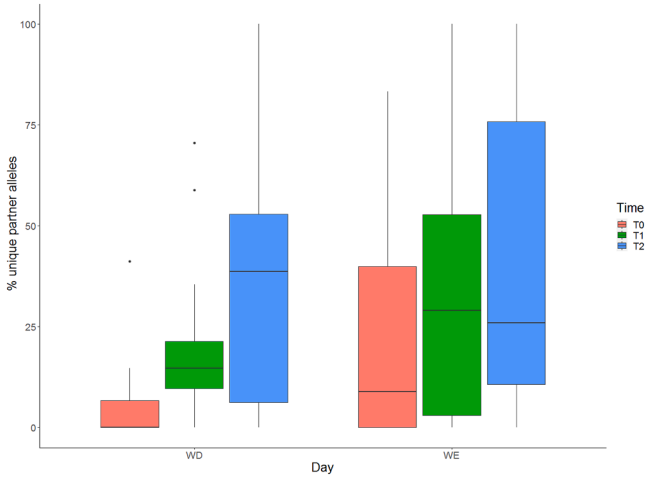

Fig. 7. Box plots displaying the percentage of unique partner's alleles detected vs the three sampling time-points (T_0 = 0 h after the shower, T_1 = 7–8 h after the shower and T_2 = 24 h after the shower) and the two sampling days (WD = weekday, WE = weekend).

(Fig. 7).

3.12. DNA from unknown contributors

Unknown alleles are defined as alleles that are not derived from the sample donor, the partner or their children. Nine percent of the samples (n = 11) displayed unknown alleles. Table 2 shows the number of samples with the maximum, minimum and the average number of unknown alleles detected over time, on the two sampling days and for different living arrangements.

3.13. Questionnaire and activities evaluation

The activities questionnaires delivered by each participant were analyzed comparing the time and day of an activity, to the two sampling days and the three sampling time-points. Not all participants provided detailed information on the questionnaire. Only the activities reported with precise timing and day records were examined. Samples from participants in a relationship that did not provide the partner's reference sample were not taken into account when using the number of unknown alleles for activities evaluation. An activity was considered as performed if it happened before collecting a sample. Details on the activities questionnaire results are reported in Supplement S3, Table S3.

Table 2
Number of samples and number of unknown alleles detected over time, on the two sampling days and for different living arrangements. T_0 = 0 h after the shower, T_1 = 7–8 h after the shower and T_2 = 24 h after the shower; WD = weekday, WE = weekend.

	N samples	N unknown alleles (min-max, mean)
Time:		
T_0	0	–
T_1	5	2–6 (mean = 4)
T_2	6	1–12 (mean = 4)
Day:		
WD	6	1–12 (mean = 5)
WE	5	1–6 (mean = 3)
Living arrangement:		
alone	1	6
flatmates	0	–
partner	4	2–6 (mean = 4)
partner & children	5	1–12 (mean = 5)
partner & flatmates	1	2

3.13.1. Use of public transport

The number of unknown alleles was compared in relation to the use of public transport. Thirty-one percent of the samples from the participants that declared to have taken public transport (n = 16) displayed unknown alleles compared with 7% of the samples from the participants that declared to have not taken the public transport (n = 84). The sample containing the highest number of unknown alleles was collected from a participant that used the public transport before collecting the sample.

3.13.2. Attendance of public spaces

The number of unknown alleles was compared to the attendance of public spaces. Sixteen percent of the samples from the participants that declared to have attended a public space (n = 31) contained alleles from an unknown source; nine percent of the samples from the participants that declared to have not attended public spaces (n = 69) also displayed unknown alleles. The sample containing the highest number of unknown alleles was collected from a participant that declared to have attended a public space (gym), before collecting the sample.

3.13.3. Social contacts

The number of unknown alleles detected was compared to social contact events with unrelated individuals, reported by the participants in the activities questionnaire. Twelve point five percent of the samples from the participants that declared to have had social contact before collecting a sample (n = 16) presented unknown alleles, compared to 11% of the samples from the participants that declared to have had no social contact before sampling (n = 81), i.e. very little difference between the two sets.

3.13.4. Use of cosmetic products

The use of cosmetic products was analyzed in relation to the total number of alleles detected and to the DNA-quantity. A total of 13 samples were collected after the use of cosmetic products (e.g. face cream, moisturizer, serum and perfume) on the sampling area. No significant difference was found in the total number of alleles between the samples collected after the use of cosmetic products and the samples collected without having applied cosmetic products on the sampling area (n = 103). The samples collected after using cosmetic products displayed lower DNA quantities than the samples collected when no cosmetic products were used, however the difference was not statistically significant (Wilcoxon, p = 0.4).

3.13.5. Use of unwashed clothing or items

A higher number of non-self alleles were detected from the samples of the participants that declared to have worn unwashed clothing or items on their neck, before collecting the samples (n = 48), than from the samples of the participants that did not wear unwashed clothing or items over the sampling area (n = 55). However, this result was not significant (Wilcoxon, p = 0.2).

4. Discussion

4.1. DNA-quantity and interpersonal differences

The DNA-quantity of the samples collected from the neck showed both inter- and intra-personal variations. Only one sample generated no profile (0 alleles detected at 100 RFU) and 17 samples resulted in partial donor profiles (with or without foreign alleles). The variability in the amount of DNA collected could be related to the shedder status of the individuals (sample donor, partner and unknown contributors), but also to the different abilities of each participant to collect the samples. Participants were asked to collect their samples at the established time-points by applying a moderate pressure on the neck surface. Most of the volunteers of this study were trained forensic biologists; however each individual may have applied a different pressure on the neck when collecting the samples. The shedder status of the participants was not tested by the traditional method (DNA amount deposited by hands). However, the analysis of the differences in donor contributions showed that two participants could be distinguished among others for their low donor contribution and two for their high donor contribution. Fonneløp et al. [11] and Goray et al. [12] observed that high shedders were typically associated with fewer contributors in samples collected from their T-shirts and hand prints, respectively. The contrary was observed for low shedders. Non-self DNA transferred to a high shedder may be masked by the high amount of his/her own DNA [11,12]. The shedding ability of an individual could have influenced the quantity of non-self DNA that was recovered from his/her skin and it is expected that the donor contribution corresponds to the shedder status of the individual. Goray et al. [24] however showed that the shedder status on other parts of the body may differ from that found by collecting samples from hand deposits. The number of non-self alleles detected from different participants was compared to their donor contributions; from one of the participants with a high donor contribution only one sample displayed partner's alleles. This differs from what was observed from the samples of other individuals living with the partner, where on average a high number of partner's alleles were detected. The second individual with a high donor contribution displayed no unknown alleles; however, this person was also living alone. Considering the two participants displaying a low donor contribution the results were not as expected; both individuals showed a low number of non-self alleles compared to the average. The shedder status of sample donors could have influenced the recovery of DNA from their neck; nevertheless, other factors such as the sampling process, the living arrangement, the shedder status of the partner and unknown contributors, and the behavior of the individual before collecting the samples may have had a greater impact on the results of this study. Hair length is another factor that could have had an impact on the differences observed between individuals. A small increase (not significant) in the number of non-self alleles detected was observed for samples collected from male participants compared to samples from female participants. However, in this study the sample donors' gender may not be a suitable approach for evaluating the effect of hair length on non-self DNA levels and composition on the neck. Indeed, some of the female participants had short hair or declared to have been using a hair band during the sampling days. Further investigations, by a controlled study, would be necessary to study how hair length affects non-self DNA present on the neck.

4.2. Control study and sampling areas

Three different sampling areas on the neck were used to monitor changes over time. It is possible that the location on the neck also influences the amount and composition of DNA detected. The control study was performed to determine if, at T_0 (just after showering), there were differences in DNA-quantity recovery from the three areas of skin. Different amounts of DNA were detected from the three participants and neck areas; two of the participants showed the highest amount of DNA in the samples collected from the sides of the neck while the third participant displayed the highest DNA-quantity on the front of the neck. The samples collected from the back of the neck resulted in partial donor profiles and showed the lowest amount of DNA for two of the participants. Samples from the sides of the neck were collected from two skin surfaces and it is possible that a larger area than in the front and back could have been sampled by some individuals. Moreover, participants could have encountered difficulty in collecting a DNA sample from swabbing the back of their own neck and this may have resulted in less DNA collected from this area. The variations observed in the DNA-quantity for the three sampling areas, from the small dataset of samples collected for the control study, did not have the same impact when considering all data from the main study. On average, no significant differences were observed in the amount of DNA detected at the three sampling points. Although the DNA-quantity did not vary, this study

showed that the number of non-self alleles and the mixture proportions from samples collected from the neck change over time.

At T_0, 42% of the samples were mixtures but only few non-self alleles and low non-self DNA mixture proportions were observed. In the main study, only one sample (collected at T_0 in a weekday) from one of the participants of the control study was a mixture with just two partner's alleles. The corresponding samples from the other two participants of the control study resulted in single donor profiles. The findings of the main study for T_0 samples correspond to the results of the control study, where no non-self DNA was detected in any of the samples from the three participants.

In the main study, the back of the neck was sampled at T_2 (24 h from showering). T_2 samples displayed the highest number of non-self alleles detected over the three sampling time-points (and sampling areas). On the contrary, the control samples collected at T_0, from the back of the neck, did not show any non-self alleles and resulted in partial donor profiles. The difference observed from the samples collected from the back of the neck at T_0 (control study) and the samples collected from the back of the neck at T_2 (main study) suggests that the sampling area may have had only a minor influence on the results of this study. The accumulation of DNA on the neck over time is more likely the main factor that caused the difference observed between samples collected at the three time-points.

4.3. Mixtures and sample contribution

More than half (62%) of the samples analyzed resulted in DNA mixtures of two (85%) or three (15%) contributors. Only 38% of the samples resulted in single donor profiles. This confirmed that foreign DNA is normally present on the neck of adult individuals at levels detectable with standard forensic methods [2,4]. The number of DNA mixtures increased with time from T_0 to T_2, showing that non-self DNA accumulates on the neck over the day. The analysis of sample contribution over time represents a comprehensive picture of the results of this study. The contribution from the sample donor (C1) decreased with time from T_0 to T_2. The contribution from the partner/second contributor increased with time from T_0 to T_2. Unknown contributors were detected only at T_1 and T_2, with a higher contribution displayed by the samples collected at T_1. In this study we observed that self DNA present on the neck tended to be complemented by non-self DNA that increased over the course of 24 h, if an individual did not take a shower or washed the sampling area. Most of the foreign DNA detected on the neck could be associated with the partner and/or children (when present) and only a small amount of DNA was derived from unknown sources.

4.4. Non-self and partner's DNA

An increase in the number of non-self alleles was observed over time, with the samples collected at T_2 presenting the highest number of non-self alleles. The highest percentage of donor-partner mixtures was observed for T_2 samples, followed by samples collected at T_1. A lower percentage of samples collected at T_0 resulted in two-person mixtures of sample donor and partner. Importantly, no unknown alleles were detected in any of T_0 samples. A shower should negatively influence DNA persistence on skin. The detection of partner's DNA on the neck just after a shower may be related to the way the participant washed his/her neck (e.g. scrubbing the skin vs not scrubbing the skin) or may have happened because DNA from close family members (e.g. partner and children), present in the shared bathroom, was transferred on the neck just after the shower. Participants were asked to dry themselves with a clean towel. The possibility that participants did not use a clean towel needs however to be considered. In addition, DNA from household members can be present on towels washed in a shared washing machine, due to secondary DNA transfer during laundry. DNA may also persist in the towel from a previous user or be transferred by the person that stored the towel after the laundry [25]. The possibility of DNA transfer from the towels cannot be excluded since no background samples were collected from the towels used by each participant. The presence of children's alleles in DNA mixtures from the parent was inferred by the LR_{rel} value only for one sample. However, alleles from children might also be present at low level in other mixtures and masked by parents' alleles, so that their presence could not be inferred by LR_{rel}. A higher number of mixtures were detected from samples collected during the weekend than from those collected during the weekday. Sixty-two percent of the samples collected during the weekend resulted in two-person mixtures. The partner was the minor contributor in all but two mixtures. Variable amounts of non-self DNA can be normally found on the neck of cohabiting partners, where the partner is not necessarily a minor contributor. The two mixtures displaying partner's DNA as major component were from individuals that had physical contact with the partner before collecting the sample; this indicates that, in normal conditions, it is not likely to detect partner's DNA as the major mixture component in samples collected from an individual's neck. The detection of partner's DNA on the neck at high levels may then suggest that very recent direct physical contact(s) may have happened. The percentage of unique partner's alleles detected increased significantly over time. This can be explained with the accumulation of partner's DNA on the sample donor's neck occurring throughout the day. Malsom et al. [14] showed that the incidence of mixed DNA profiles detected from fingernail samples increases as a couple spend increasing amount of time together. Gill et al. [16] stated that partner's DNA accumulates onto the skin of a subject and that a steady state will be achieved after a period of time (c. 12 h), where the DNA amount that is gained balances the DNA-quantity that is lost. From our study we observed that partner's DNA accumulates on the neck over the course of 24 h. Partner's DNA was consistently detected in T_1 samples, both from the samples collected on the weekday and from those collected over the weekend. T_1 samples from the weekday were collected when the participant was still at the workplace. Thus partner's and/or children's DNA persists on the neck also when the individual has spent several hours away from the shared home environment with no contact with his/her partner and children. This can be explained by partner's and/or children's DNA being transferred on the sample donor's neck in the morning, before the participant went to work or by DNA transfer from the clothes worn by the participant, which may have contained DNA from the partner and children. The recovery of DNA traces from clothing has been investigated by several authors. Szkuta et al. [26] conducted an inter-laboratory study on worn upper garments and found that DNA from cohabiting individuals can be transferred on internal or external areas of clothing, and may persist for a long time. In the study of Szkuta et al. [26], partner and children's DNA was frequently identified in samples collected following workday activities. Apart from possible direct transfer, the shared home environment, communal washing, drying and storage of clothing items was used as an explanation of indirect DNA transfer from partner and children to the clothes [25,26]. All participants with partner and children except one, for which there were missing information in the questionnaire, declared to have spent a significant amount of time with their family during the weekend. This is consistent with the higher percentage of partner's alleles detected in the samples collected over the weekend. A limitation of this study is that three participants did not provide their partner's reference sample. For the samples collected from these participants, all alleles not matching the donor were counted as one, since heterozygote and homozygote alleles could not be distinguished, leading to underestimation of the total allele count. These samples were also excluded from the evaluation of unique partner's alleles since there was no certainty on the origin of the non-self alleles; this may have lowered the statistical strength of the study.

4.5. DNA from unknown contributors

DNA from unknown contributors was detected in 11 samples, but none was collected at T_0. Conversely, partner's (and/or children's) DNA

on the neck was often found at T_0; this suggests that a close relationship and cohabitation may be necessary to explain the presence of non-self DNA on the neck just after a shower. All DNA mixtures that displayed unknown alleles were detected either in T_1 or T_2 samples, both weekday and weekend. All the participants from whom these samples were collected declared to have spent time in a public space (e.g. workplace, gym, shop), have used unwashed clothing/items, have taken public transport and in one case to have spent time with friends before collecting the samples. The DNA profile with the highest number of unknown alleles was from an individual that used public transport and attended a public space (gym) before collecting the sample. Unknown DNA components may have been directly transferred from the persons the participant interacted with (e.g. colleagues, friends) or indirectly transferred from the surrounding environment attended by the individual (e.g. public transport, gym). Regardless of time, day, living arrangement and activities, a small number of unknown alleles ($\leq$12) were detected. This suggests that detecting an unknown individual as a full or major profile, from samples collected from the neck, could strongly indicate the occurrence of direct physical contact(s). Only 9% of the samples analyzed presented unknown alleles. This may be the result of the strict COVID-19 restrictions at the time of the experiment. A social distance of minimum 2 m was recommended between people and the use of a face mask if this was not possible. This may have reduced direct DNA transfer between individuals. The use of face masks may also have decreased the amount of aerosol DNA that may have been transferred to the neck of two persons talking to each other and the DNA contamination of surfaces due to speaking [27,28]. Moreover, the use of hygienic measures such as frequent hand washing and the disinfection of surfaces and items in public spaces may have influenced the results of this research. The sampling skin area could itself be a reason for the low amount of DNA from unknown sources detected. Szkuta et al. [26] suggested that the transfer of non-self DNA is minimized in internal garment areas such as the collar and cuff, because these areas are not in direct contact with the surrounding environment. The neck is protected from the outdoor environment and typically covered by clothes and hair; therefore less DNA is likely to be transferred on this skin area. Different parts of the body may contain a different amount of non-self DNA, dependent upon relative exposure to the outdoor environment.

4.6. Living arrangements of sample donors

The living arrangement of an individual seemed to have an effect on the composition and level of non-self DNA present on his/her neck. All of the samples, except one, from the participants living alone resulted in single donor profiles. Surprisingly all of the samples from participants sharing the house with flatmates also resulted in single donor profiles. This suggests that cohabitation is not enough to explain the presence of non-self DNA on the neck surface. A similar finding was observed by Matte et al. [29] who studied the prevalence and persistence of foreign DNA beneath fingernails. Fingernail samples were collected from co-habiting students and although 14% of the collected samples contained foreign DNA, none of the unknown profiles detected could be attributed to a household member. The presence of non-self DNA on the neck is more likely to be associated with a close relationship and physical contacts between individuals living in a shared environment. This is especially true when considering the conditions in which the study was performed. Due to COVID-19 restrictions, non-related persons were asked to keep safe distance, whilst related individuals most likely behaved normally. This could be one of the explanations for the absence of foreign DNA on the neck of the individuals cohabiting with flatmates. Participants cohabiting with the partner showed a high number of non-self alleles due to the accumulation of partner's alleles on the neck. The same result was found from a study of Graham et al. [4] where most of the non-self DNA was detected on the neck of volunteers married and/or living with partner. Cohabiting partners that shared a common household were found to carry each other's DNA and cohabitation affected non-donor DNA obtained from fingernail swabs [14,30]. An even higher number of non-self alleles were detected from the samples of participants cohabiting with partner and children. Children tend to transfer more DNA than adults [10,31]; this may explain the higher number of alleles detected from the samples of the participants cohabiting with partner and children and confirms that alleles from both partner and children may be present in these samples. DNA from cohabiting partners and children is transferred, accumulates and persists on the neck the whole day. The highest number of non-self alleles was detected from the samples collected from two participants cohabiting with partner and flatmates. However, only one of these samples presented unknown alleles, while the others were single donor profiles and mixtures of donor and partner. Thus, the high number of non-self alleles consisted of partner's alleles rather than alleles from unknown contributors. In this study, a small number of individuals were present in three of the living arrangement's categories: "alone", "flatmates" and "partner & flatmates". Further investigations, with a higher number of participants for those categories, would be beneficial to confirm the impact that different living arrangements may have on the composition and levels of non-self DNA on the neck.

4.7. Activities questionnaire evaluation

All of the 11 samples that displayed unknown alleles were from participants that: used the public transport, attended a public space, had social contacts or used unwashed clothing or items before collecting the samples. These activities seemed to enhance the chance that DNA from unknown sources was transferred to the neck. DNA profiles from known and unknown persons can be detected on the neck of a victim due to normal DNA transfer occurring throughout daily life. No significant effect was observed in the DNA concentration and total number of alleles for the use of cosmetic products before sampling and the use of clothing or items not recently washed.

5. Conclusion

Non-self DNA is normally present on the neck surface of adult individuals due to DNA transfer during daily activities. We showed that non-self DNA tended to accumulate on the neck over the course of 24 h. DNA from partner and/or children was detected just after an individual took a shower, increased throughout the day and persisted when the individual spent several hours away from the shared home environment. We found increasing amounts of DNA from cohabiting partners over time and a higher percentage of donor-partner mixtures was detected in samples from the weekend in contrast to the weekday. The living arrangement and relationship status of an individual had an effect on the composition and levels of non-self DNA present on the neck. We showed that participants cohabiting with partner and children displayed high levels of non-self DNA in comparison to single participants living alone. However, cohabitation is not enough to explain the detection of non-self DNA on the neck since individuals cohabiting with flatmates only displayed their own DNA profile for all samples. Hence, the presence of non-self DNA on the neck is more likely associated to a close relationship and physical contact between persons in the same household. Moreover, the detection of non-self DNA on the neck as a major mixture component may suggest increased and direct physical contact between individuals. When partner's DNA is collected from the neck of a victim, this need to be carefully evaluated, together with the specific case circumstances, since variable amounts of partner's (and/or children's) DNA are normally present on the neck surface. DNA from unknown individuals can be transferred to the neck from the surrounding environment, for example when a subject uses public transport, attends public spaces and interacts with other persons. The results of this study will aid in the evaluation at the activity level of DNA evidence collected from the neck of a victim.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2022.102661.

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