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Forensic analysis of fingernail debris after a scratch experiment and its applications in violence against women investigation

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Abstract

In violence against women investigations, the collection of fingernail debris may provide valuable biological evidence for DNA analysis to allow the identification of the perpetrator and the corroboration of criminal circumstances. Indeed, in both sexual and non-sexual assaults against women, mainly perpetrated by males, the close and violent physical contact between the victim and assailant may lead to the accumulation of genetic material under the protected fingernail hyponychium area, which can be analysed for DNA typing for court proceedings.

In this context, the main objective of the present study was to explore the mechanisms of male DNA transfer, persistence, and recovery under fingernails of women following a mock physical assault, as well as the prevalence of male cellular material under normal, non-criminal circumstances. The study aimed at comparing non-criminal everyday male DNA transfer beneath fingernails and transfer under mock criminal circumstances to identify factors that may have led to the detection of foreign DNA profiles in the two possible scenarios. This information might be helpful for linking forensic sample collection with the molecular genetic findings from fingernail samples and aid the assessment of the genetic evidence's value by the court.

Forty-eight female volunteers were enrolled for a controlled scratch study and asked to superficially scratch male volunteers' forearms, mimicking a defensive action during a physical assault. To set a defined baseline regarding the genetic background signal, scratching took place after the cleaning of the hyponychium area. Targeted sampling of fingernail debris was performed at regular time intervals after the scratching to explore the persistence of male cellular material over time and in relation to factors that may have affected its detection. In addition, fingernail samples were collected from female volunteers two weeks before the scratch experiment, to investigate the prevalence of male DNA under volunteers' fingernails in normal daily life.

A total of 288 pre-experimental fingernail samples (PES) were collected from female volunteers for the prevalence study and, for the transfer and persistence study, a total of 288 fingernail samples were collected after scratching (NS). The transfer, persistence and prevalence of male DNA under volunteers' fingernails were investigated through a Y-STR typing approach and complemented by autosomal STR profiling of relevant samples. For the recovery study, two different sampling strategies were employed, involving either cumulative or finger by finger sample collection.

The prevalence of male DNA under volunteers' fingernails revealed by the persistence study was 37.5% (n=108), with 17.7% (n=51) of the entire PES set yielding useful Y-STR profiles. Of these, 39.2% (n=20) were of good quality, while the remaining 31 samples produced low template Y-STR profiles. Among the useful Y-STR profiles, 56.9% (n=29) matched the volunteers' partner or son's Y haplotype, corresponding to 10.1% of the entire PES set. Being involved in an intimate relationship, cohabiting with males, the use of

shared objects, bed-sharing and activities involving holding hands, were positively associated with the finding of partners' or sons' Y haplotype.

In the transfer and persistence study, useful Y-STR profiles were obtained from 73.6% (n=53) of the fingernail samples collected immediately after scratches (t=0), of which 58.5% (n=31) showed a good profile quality. The Y haplotype of the scratched male was found in 88.7% (n=47) of the useful Y-STR profiles, corresponding to 65.3% of the entire t=0 sample set. However, with autosomal profiling, this proportion dropped to 69.8% (n=37). In total 20.8% (n=11) of the autosomal STR based exclusions of the scratched male as a possible contributor to the sample occurred due to the simultaneous absence of the scratched male's alleles at some loci and the presence of drop-in alleles, possibly deriving from a third contributor. This situation was further complicated by allelic dropouts affecting the female volunteer's profile. Nevertheless, for 2 samples the scratched male was detected by autosomal profiling, whereas Y-STR analysis led to his exclusion.

When the sampling of fingernail debris occurred 6 (t=6), 12 (t=12) or 24 (t=24) hours after scratching, useful Y-STR profiles were obtained at distinctly lower rates: 16.7% (n=12) of the 72 t=6, 23.6% (n=17) of the 72 t=12, and 27.8% (n=20) of the 72 t=24 samples. Likewise, also the number of good quality profiles dropped to values of n=3 (25.0%), 7 (42.9%) and 13 (65.0%) of the useful profiles for t=6, 12 and 24 respectively. Interestingly, the Y haplotype of the scratched male was found only in 2 t=6 samples, in 2 t=12 samples and in another 2 t=24 samples, which accounted for 2.8% of the whole t=6, t=12 and t=24 sample sets. By autosomal STR profiling, the scratched male's profile was observed in one t=6 sample, in two t=12 samples and in zero t=24 samples. Remarkably enough, in t=12 and t=24 samples there has been a rise in the detection of partners and cohabitant Y haplotypes and autosomal profiles, a finding that might have been associated with female volunteers' activities involving spending time with the male partner and the use of shared objects.

The recovery study indicated that the cumulative collection method results in higher amounts of male DNA being collected. However, for samples collected after scratching this difference was not necessarily of utmost practical relevance, as good quality profiles were also obtained from samples collected from single fingers.

In general, the findings of this study suggest that the amount of male cellular material acquired under fingernails through scratching greatly decreases over time and it can be lost during normal daily-life activities. Therefore, in violence against women investigations, the collection of fingernail samples should be performed in a finger by finger fashion as soon as possible to increase the chances for obtaining meaningful results, although relevant profiles were also obtained from samples collected 6, 12 and 24 hours after the scratching. Nevertheless, an increased time lag between scratching and sample collection results in a higher chance to obtain genetic profiles acquired from normal contacts, as non-crime related DNA transfers may occur under fingernails. This is particularly true for the victim's intimate partner, which complicates the overall assessment of the genetic findings.

Introduction

Violence against women is a pervasive phenomenon affecting women and girls worldwide, as well as a gross violation of women's human rights with a deep impact on society. The most common forms of violence experienced by women throughout the world are intimate partner violence and sexual violence, and almost one-third of all women who have ever been in a relationship have experienced physical and/or sexual violence by their intimate partner.

Violence against women results in significant consequences for the physical and psychological health of victims. Therefore, comprehensive and gender-sensitive health and legal services for the emergency management of survivors are imperative to cope with health consequences and women needs while providing adequate protection. As part of the emergency management of victims of gender-based violence, the medico-legal investigation occupies a central role in documenting signs of the violent act, defining the manner of death in case of femicide, and in collecting the relevant forensic evidence for corroborating the circumstances of the alleged assault and identifying the perpetrator.

During sexual and non-sexual assaults, violent physical contacts between victim and assailant may determine the transfer of biological materials, which can ultimately provide the assailant's genetic profile. Actually, DNA testing is an established and highly probative part of the investigation and prosecution of both sexual and non-sexual assaults against women, allowing for establishing the occurrence of an alleged assault, for corroborating its circumstances and for identifying assailants.

DNA can be obtained from various sources of biological material found in sexual and non-sexual assault investigations and even trace samples may provide valuable probative evidence of the crime, assisting in the identification of the suspect, particularly when no penetration or sexual attempts have been reported.

However, since trace DNA usually cannot be attributed to a biological source, to assess the biological results given the alleged activities, the significance of findings needs to be based on information about factors that affect DNA transfer and persistence on the specific sampling area. Moreover, the presence of cellular material from more than one person may lead to complex mixed DNA profiles, further complicating the interpretation of genetic results in sexual and physical assault investigations. This is particularly the case with samples collected under fingernails of women after a physical struggle in sexual and non-sexual assaults investigations, where trace amounts of foreign DNA are usually found in a high background of female DNA.

The fingernail hyponychium is an isolated and protected area in which trace amounts of skin and biological fluids may accumulate, hence providing evidential biological material for genetic investigation. However, when evaluating foreign DNA profiles obtained from fingernail debris, DNA prevalence, transfer and persistence under fingernails, as well as normal everyday and crime-related activities need to be assessed to interpret the significance of the forensic genotyping results and present the evidence in court.

Within this frame, the aim of the present thesis is to investigate the mechanisms of DNA transfer, persistence, prevalence and recovery under fingernails of women following a mock physical assault, by conducting a controlled scratch study in which female volunteers scratch male volunteers.

Variables that may affect the detection and the persistence of male DNA profiles under fingernails in normal non-criminal circumstances as well as in mock criminal scenario were investigated by means of questionnaires, as personal habits, daily activities, activities performed since scratches, hygienic habits and human contacts in the previous 48 hours.

Providing information on the level of contact required for the transfer of cellular material beneath female fingernails and on factors that affect DNA transfer and persistence under mock criminal circumstances may assist in the interpretation of DNA profiles obtained from fingernail samples in cases of sexual and non-sexual assaults against women. In addition, discriminate between normal everyday DNA transfers and crime-related transfers may allow assessing the relevance of foreign DNA profiles found under fingernails given the alleged activities, particularly when the victim and offender are known to each other, are in a relationship and/or live together.

Lastly, since there are several factors that can influence the amount of DNA recovered from under a fingernail, another objective of the present study is to compare two different collection strategies, i.e. cumulative and finger-by-finger, to provide valuable insight and develop best practices for fingernail evidence forensic analysis.

Violence against women

1.1 Definition

Violence against women is a complex and multi-dimensional phenomenon that results in a significant public health problem, as well as in a gross violation of women's human rights.

The Declaration on the Elimination of Violence Against Women [1], adopted by the United Nations General Assembly in 1993, defines violence against women as “*any act of gender-based violence that results in, or is likely to result in, physical, sexual, or mental harm or suffering to women, including threats of such acts, coercion or arbitrary deprivation of liberty, whether occurring in public or in private life. Violence against women shall be understood to encompass, but not be limited to, the following: (a) Physical, sexual and psychological violence occurring in the family, including battering, sexual abuse of female children in the household, dowry-related violence, marital rape, female genital mutilation and other traditional practices harmful to women, non-spousal violence and violence related to exploitation; (b) physical, sexual and psychological violence occurring within the general community, including rape, sexual abuse, sexual harassment and intimidation at work, in educational institutions and elsewhere, trafficking in women and forced prostitution; (c) Physical, sexual and psychological violence perpetrated or condoned by the State, wherever it occurs*”.

According to the United Nation's definition, the term violence against woman refers to any form of interpersonal gender-based violence. It includes acts of physical aggression, female genital mutilation, forced marriage and so-called honour-based violence, sexual abuse, sexual assault, trafficking and prostitution, psychological abuse, harassment, coercion and controlling behaviours, which can be overall summarized into three main categories: physical violence, psychological violence and sexual violence. Among these, physical and sexual assaults are the most prevalent forms women experience worldwide [2]. Acts of violence can occur within the family being perpetrated by a family member and generally referred as domestic violence, and more specifically, they can be perpetrated by a current or former intimate partner and defined as intimate partner violence, as well as unknown individuals, named non-partner violence.

The direct and indirect health consequences of the violence against women may range from manipulation, psychological damages and emotional disorders to injuries and traumas generated by the violent acts, up to femicide (also known as feminicide), the most extreme form of violence against women.

All these forms of violence and abuse, disproportionately committed against women and girls, are driven by profound inequalities between women and men, connoting an unbalanced relationship between the genders in which the perpetrator of the violence, usually male, asserts itself over the woman through physical, psychological and/or sexual imposition. Power and authority are key motivations for violence against

women, violent acts are motivated by domination, exclusion, control, possession and repression, and individual acts of violence are supported by a context of structural inequality and cultural misogyny further sustained by patriarchal power relations [3].

1.2 Global and Italian prevalence

The World Health Organization (WHO) [2] reported that worldwide 35% of women have experienced in their lifetime either intimate partner physical and/or sexual violence or non-partner sexual violence, or both. Intimate partner violence and non-partner sexual violence are widespread and are the most common forms of violence experienced by women throughout the world. Almost one-third (30%) of all women who have ever been in a relationship have experienced physical and/or sexual violence by their intimate partner. There is considerable regional variation in the prevalence of physical and/or sexual partner violence, ranging from 23.2% in high-income countries and Western Pacific region to 37% in the WHO Eastern Mediterranean region and South-East Asia region (*Fig. 1*), up to 71% in Ethiopia province [4].

In addition to intimate partner violence, globally 7% of women, ranging regionally between 3.3% and 21%, have been sexually assaulted by someone other than a partner, although data for non-partner sexual violence is more limited [2].

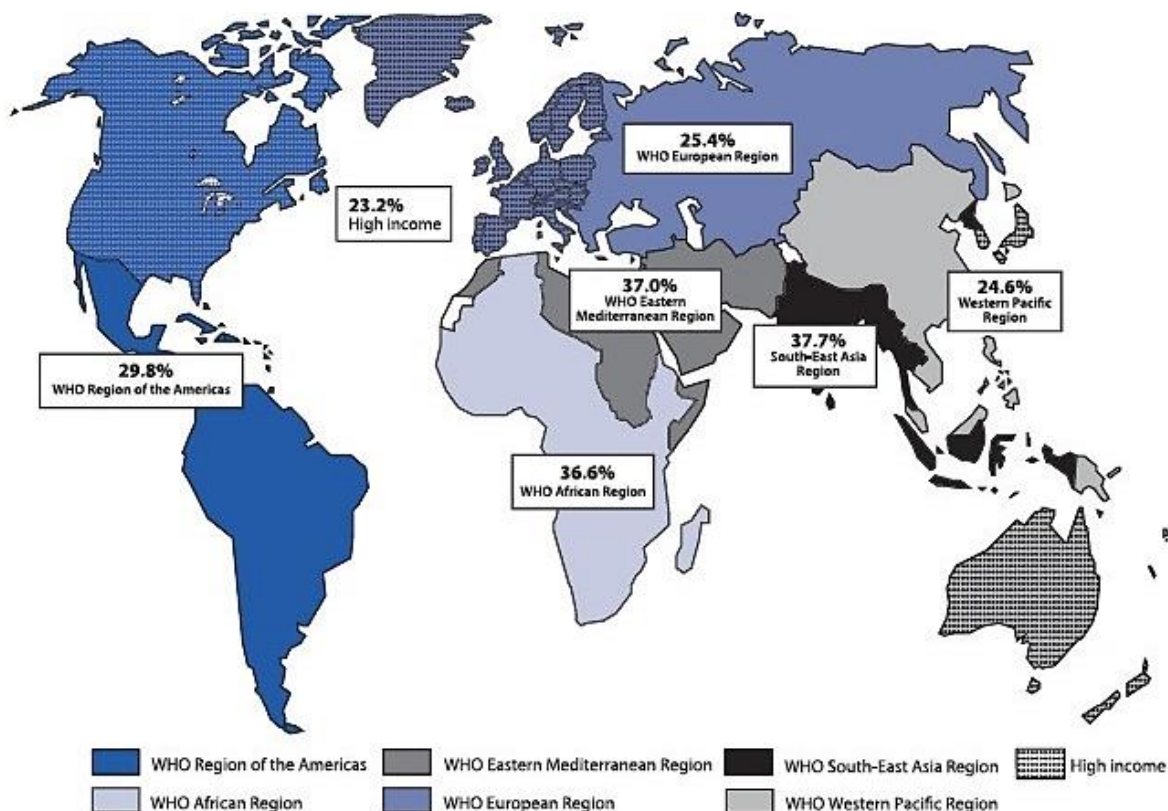


Figure 1: WHO's regional prevalence rates of intimate partner violence (2010). Figure from: *Global and regional estimates of violence against women: prevalence and health effects of intimate partner violence and non-partner sexual violence*. Geneva: World Health Organization; 2013 [2].

Intimate partner violence accounts for a high proportion of homicides of women all over the world: globally between 38% and 55% of femicides are committed by male partners, while the corresponding number for men is 6%. Moreover, 42% of women who have been physically and/or sexually abused by a partner have experienced injuries as a result of that violence [2].

In Italy, according to the latest ISTAT survey [5], 31.5% of the women between 16-70 years of age have been victims of physical and/or sexual violence; 20.2% have suffered physical violence, 21% sexual violence and 5.4% more severe forms of sexual violence such as rape and attempted rape. Among these, 13.6% have been victims of physical or sexual violence by their current or former partner while 24.7% have been physically or sexually assaulted by a non-partner, including 13.2% of unknown individuals. In addition, during the year 2017, 80.5% of femicides in Italy were mainly committed within the family by men known to the victim: 43.9% were perpetrated by the current or former intimate partner, 36.6% by a relative or an acquaintance, while 6.5% by an unknown person [6]. Furthermore, while recently there has been a constant reduction in the homicide of males, the rate of femicide remains stable [6].

The growing body of research in this area has revealed that violence against women is a widespread phenomenon affecting women throughout the globe. Across regions, intimate partner violence is considerably more prevalent and more common than non-partner sexual violence; however, both forms of violence remain highly stigmatized in society, therefore are likely to be significantly underreported [7]. In addition, data on intimate partner homicide and other types of femicide are provided at the national level and may differ from country to country due to the differences in reporting and registration procedures, hence the actual prevalence may be underestimated [8].

Femicide is the most extreme consequence of violence against women and it is often the result of other forms of disproportional violence that women experience in their life. Intimate partner and family-related killings are the main causes of female homicides and, very often, femicide can be considered the epilogue of a *continuum* of existing violence inflicted by a partner, ex-partner, or other relatives that leads to women's death [8].

1.3 International standards toward the elimination of violence against women

Despite the magnitude of the problem, violence against women was, until relatively recently, considered a private matter in which the State played only a limited role. It is only since the 1990s that violence against women has been recognised as a fundamental rights' violation requiring legal and political concern at the highest level, where State Parties, in compliance with the duty to protect, have an obligation to safeguard victims. Hence, the elimination of violence against women has become an absolute priority on the political agenda of governments and international human rights organizations, including the United Nations (UN) and its specialized agency World Health Organization (WHO), European Union institutions and non-governmental organizations (NGO).

The international community has started to address violence against women as gender-based violence with the 1979 Convention on the Elimination of all Forms of Discrimination against Women (CEDAW) [9] and the Declaration on the Elimination of Violence against Women [1], acknowledging that it is an intolerable violation of women's fundamental and political rights needing urgent actions.

The growing recognition of violence against women as human rights concern was further emphasized through several international agreements and declarations at key international conferences during the 1990s, including the World Conference on Human Rights (Vienna, 1993) [10], the International Conference on Population and Development (Cairo, 1994) [11] and the Fourth World Conference on Women (Beijing, 1995) [12], which have led to the production of documents and recommendations guiding Member States to implement legislative measures to prevent and respond to violence against women toward its elimination.

The first international legally binding tool addressing more specifically the prevention, protection, prosecution and elimination of all forms of violence against women and domestic violence was the 2011 Council of Europe Convention on Preventing and Combating Violence against Women and Domestic Violence [13], known as the Istanbul Convention. Specifically, this Convention covers in detail different forms of violence, such as psychological violence, stalking, physical violence, sexual harassment and sexual violence, including rape within marriage and genital mutilation, urging on Member States for the adoption of legislative tools to exercise due diligence to respond, prevent, investigate, protect from and persecute the most severe and widespread forms of gender-based violence. Ratification of the Istanbul Convention committed States Parties to take measures for opposing to violence against women in all fields, including the adoption of specific legislation in national legal systems and penal codes, and their implementation monitoring.

1.4 The Italian legal framework

The Italian Government has ratified the Istanbul Convention under the Law n. 77 of June 2013 and has adopted Decree-Law 93/2013 then converted into Law n. 119 of October 2013, for combating violence against women, containing, *inter alia*, measures aimed at reinforcing the protection of women victims of violence, at preventing the phenomenon and establishing more rigorous punishments for perpetrators. However, significant legal norms against gender-discrimination have been previously introduced, passing through different steps as the initial sign of social and legal changes. From a regulatory point of view, only in 1981 was the law changed to end the granting of reduced sentences in cases of so-called "honour killings" perpetrated to save the family's honour after a betrayal, along with the law permitting reparative marriage, which allowed men to "repair" a rape by marrying the violated woman [14].

The first significant legislative innovation in the matter of violence against women occurred with the approval of the Law n. 66 of February 1996, which recognize sexual violence as a crime against the personal liberty and no longer as an offence against public morality, innovating the previous legislation. Successively, the Italian Government passed a range of measures designed to address the problem of violence against women, including laws against prostitution, for the prevention and prohibition of female genital mutilation

practices and against domestic violence, in addition, new crimes as persecutory acts or stalking and stiffer punishments were introduced.

As part of the broader plan to combat violence against women, the Italian Parliament has recently approved (18 July 2019) the DDL “*Codice Rosso*”, which fully implements the provisions of the Council of Europe Convention on Preventing and Combating Violence against Women and Domestic Violence. The law specifically implements in the Italian penal code stiffer penalties and aggravating factors in matters of violence against women and femicide, it imposes more rapid court proceeding and introduces new crimes such as revenge porn and victim’s disfigurement, for example, by acid attacks.

With regard to prevention, Italy has predominantly implemented its commitments in this area through the adoption of two extraordinary national action plans, establishing a national observatory on violence and a national database (*banca dati*) managed by ISTAT, with the aim to collect the relevant data on acts of violence against women perpetrated across the country.

1.5 Strategies to prevent and protect women victims of violence

Over the last few decades, the international community has focused its efforts toward the elimination of violence against women through different, but overlapping and largely complementary, approaches and strategies.

International agreements were an important starting point in setting standards for national legal reforms aimed at improving the status of women, establishing specialized policies and creating a non-violent social environment. In this frame, States have adopted measures to prevent, protect and prosecute acts of violence against women to end the pattern of impunity for perpetrators of acts of violence and ensuring justice to victims.

In order to develop effective strategies to prevent the problem as well as to monitor the efficacy of the recommended interventions, international treaties have stressed the importance of collecting statistical data at regular intervals on violence against women prevalence, causes, consequences and conviction rates. Task promptly carried out by the WHO that, by promoting international research, played a key role in producing comparative data to guide policy and supervise implementation [2, 4, 7, 8, 15].

The WHO has also brought attention to the impact of violence on women’s health and the need to prevent risk factors and provide an effective response to victims once the violence has occurred [2, 4]. Despite the relevance of deaths resulting from femicides, a wide range of violence-related conditions, including sexual and reproductive disease, mental illness, post-traumatic stress disorder and chronic pain syndrome, significantly affect victims’ health. Therefore, in the effort to strengthen the health system response to gender-based violence, the WHO along with international and regional bodies have developed multi-sectoral evidence-based guidelines, standards and a global plan of action for healthcare professionals guiding the management and treatment of women victims of violence [16-19]. At the regional level, the Italian Presidency of the Council of Ministers has published the “Italian National Guidelines for Health Authorities

and Hospitals in case of First Aid and Socio-Medical Assistance for Women Victims of Violence” on February 2018 [20], guiding diagnostic and therapeutic treatments through the activation of anti-violence services.

1.6 The multidisciplinary response to violence

Comprehensive, gender-sensitive and multidimensional approaches have been suggested for the correct management of victims of gender-based violence. In this frame, the health sector copes with health consequences and women’s needs while providing adequate protection toward legal aid.

In line with international and national guidelines [16-20], the emergency management of victims of violence involves the activation of anti-violence network to provide physical and psychological support. Health professionals should ensure to survivors the required medical assistance, which may involve treatment for physical injuries and sexually transmitted diseases, pregnancy testing, emergency contraception and post-exposure prophylaxis. Moreover, a correct victim’s management requires a combination of medical and justice services with coordinated medico-legal interventions with the aim to identify, recover and preserve evidentiary material related to the sexual or physical assault to assist criminal investigations.

Evidence collection should be performed according to standardized protocols for the search, localization, documentation, and collection of all the relevant forensic traces that allow for establishing the occurrence of an assault clarifying its circumstances and identifying and prosecuting aggressors. Thus, proper handling procedures during evidence collection, packing, labelling, storage and transport, with a documented chain of custody, are imperative to guarantee reliable results and the admissibility of evidence in court [21]. Forensic evidences may be found on the victim or suspect’s bodies and clothes and/or at the crime scene, and include any items of non-biological origin such as fibres, paint, pollen, chemicals, guns and gunshot residues, as well as biological evidence such as semen, vaginal fluid, saliva, blood, sweat, urine, hair and biological debris.

As part of the investigative process, the medico-legal examination of a victim's body occupies a central role in documenting signs of the violent act, defining the manner of death in case of femicide, and in collecting the relevant samples for corroborating the circumstances of the reported assault and identifying the perpetrator. Accordingly, international guidelines have been developed as a means to strengthen the medico-legal examination, the collection and the use of forensic evidence to support investigations and court prosecutions of gender-based killings [22, 23].

1.7 Forensic medico-legal examination and evidence collection

From a legal perspective, the purpose of forensic medical examination and evidence collection in cases of violence against women is to prove or exclude a violent assault, clarify the circumstances, establish a link between the assailant and the victim or the crime scene and identify perpetrator/s. At this respect, the victim’s body may be the most important evidence, thus, the medical-forensic examination should proceed according to standard operating procedures and national protocols, and should be performed by a professional specifically trained in the collection of evidence related to sexual assault cases [22, 23].

While performing the forensic physical examination, the entire body surface, including scalp, face, neck, shoulders, forearms, arms, mouth, breasts, vagina, anus and rectum, should be carefully inspected for signs of bruises, wounds, lesions and any other injuries. Actually, violence against women may produce a huge variety of different types of lesions, ranging from bruises or stubs to permanent disability and death. The forensic examination and the subsequent interpretation of the pattern of injuries found on a victim's body, as well as on the perpetrator, may allow for drawing inferences about the nature and circumstances of the assault; therefore, detailed documentation is mandatory as it may be used afterwards in court.

During the medico-legal examination, different types of forensic evidence may be collected with the consent of the survivor to assist investigations. As stated by Locard's Exchange Principle [24], the close contact between items, individuals and places, i.e. victim, assailant and crime scene, results in the transfer of physical material and biological traces. Biological evidence is considered the most probative element in courts to prove the alleged physical contact and assist in the identification of suspects through DNA studies. However, determining which evidence needs to be collected depends upon the specifics of the case, the nature of the assault and the time elapsed since the offence. Typically, the account provided by the victim is used to guide the collection of forensic samples and may include history of injuries that the survivor may have left on the assailant's body, which eventually can be matched with the findings of the victim's examination. In addition, also information on post-assault activities may direct the sampling procedure; for example, if the victim has washed herself or had sexual intercourse with someone else since the assault, this would have a bearing on the evidence collected from that site.

The appropriate collection of evidentiary samples, which includes the report of physical examinations, record of any injuries, photographs and documentation of the collection of biological samples and evidence preservation, is essential for performing forensic laboratory analyses. Only evidence collected and analysed according to national and international standards and properly stored with a documented chain of custody may be used in court for corroborating the circumstances of the case. At the national level, the Ge.F.I. (Genetisti Forensi Italiani), Italian working group of the International Society for Forensic Genetics (ISFG), has developed guidelines for the collection of biological traces from victims of sexual and physical violence for forensic genetics analysis [25], which are included as annexe in the broader National Guidelines for Socio-Medical Assistance for Women Victims of Violence [20].

Forensic genetics and relevant human genetic variation

2.1 Forensic genetics

Since its first application in forensic casework in 1986, DNA profiling has become the gold standard evidence to be presented in court for personal identification purposes as well as for kinship testing [26].

From the discovery of DNA fingerprinting by Sir Alec Jeffreys in 1985 [27] the rapid advances in DNA technology and forensic genetic analysis have enabled its wide use in court proceedings to identify victims and convict perpetrators but also to exonerate innocents, to link a suspect to a crime scene or to a victim, to determine kinship relations and to identify mass disaster victims [26].

The forensic genetic analysis has several advantages in terms of personal identification resulting from the biological features of the DNA molecule. The DNA, or deoxyribonucleic acid, is present in the nucleus of all nucleated human cells and each cell, tissue or biologic fluid of the same individual contain the same genetic code, which does not change during time. In addition, although only a very small portion of DNA differs between individuals, these portions are highly variable both among people and between populations, allowing using DNA information for human identity purposes [26]. Forensic DNA analysis is based, indeed, on the analysis of an adequate number of variable DNA regions (i.e. polymorphisms), which allows generating unique DNA profiles differing from individual to individual, with the exception of monozygotic twins¹. This allows reaching statistical values that exceed the number of the worldwide human population, thus providing evidence for personal identification. Furthermore, since the DNA is inherited half from the mother and the other half from the father, it is useful for kinship analyses but also for indirect identification through relatives' genetic profiles.

2.2 Forensically relevant human genetic variation

Genetic variation between individuals can occur as changes to the sequence at a particular base position or as differences in the sequence length. Individuals are thought to have millions of single base changes [28, 29], which can include substitutions, insertions or deletions. Differences in sequence length generally occur in repeated segments of DNA, which make up approximately 50% of the human genome [29].

¹ Actually, there is evidence in the scientific literature that the differentiation between monozygotic twins is possible through a deeper DNA analysis, which involves the use of massively parallel sequencing approaches for the study of larger portion of the genetic code or the whole genome. However, through a standard DNA profile this differentiation is usually not possible. More details on this topic can be found in:

Budowle B. (2014). Molecular genetic investigative leads to differentiate monozygotic twins. *Investigative genetics*, 5, 11. doi:10.1186/2041-2223-5-11

Krawczak M, Budowle B, Weber-Lehmann J, Rolf B. (2018) Distinguishing genetically between the germlines of male monozygotic twins. *PLoS Genet.* 20; 14(12):e1007756. doi: 10.1371/journal.pgen.1007756.

Among DNA repetitive elements, *microsatellites*, also known as Short Tandem Repeats (STR) or Simple Sequence Repeats (SSR), are the markers of choice for human identity testing in current forensic DNA profiling practices because of their high variability between individuals and the ability to be amplified using the polymerase chain reaction (PCR). However, Single Nucleotide Polymorphisms (SNPs) have also gained interest in the forensic community. Autosomal DNA markers are required for identity purposes but, in certain cases, STRs and SNPs from the male-specific portion of the Y-chromosome and the mitochondrial DNA (mtDNA) are also investigated.

2.2.1 Short Tandem Repeats

STR loci are DNA regions characterized by a small repeat unit, usually mono-, di-, tri-, tetra-, penta- or hexa-nucleotide, which may extend for ~ 100-400 base pairs (bp) depending on the number of repetitions present. STRs are thus defined as *length polymorphisms*. The genetic variability between individuals depends, in fact, in the different number of repeat units at a certain locus, which ultimately defines the alleles of the questioned locus (**Fig. 2**).

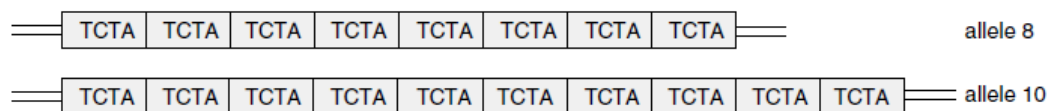


Figure 2: The structure of a short tandem repeat. Example showing the structure of two alleles from an STR locus. The core repeat is 4 bp (TCTA) and the alleles are named according to the number of the repetition of the core. Figure from: *An introduction to forensic genetics (2011) Goodwin W, Linacre A & Hadi S [30]*.

Microsatellites are widespread in the human genome, accounting for approximately 3% of the entire human genome [29]. They occur on average every 10 000 nucleotides [31] and their mutation rates are comprised between $10^{-2}/10^{-4}$ per locus per generation [32]. A major source of mutation involves increases or reductions in the number of repeat units, by a mechanism known as slippage, which occurs during the DNA replication and is facilitated by the repetitiveness of these sequences [26].

STRs suitable for forensic identification need to be highly polymorphic and heterozygous in the population of interest, to effectively discriminate between individuals, but they have to show a low mutation rate to allow for kinship analyses. Tetra-nucleotide repeats with narrow amplicon size range and moderate allele range are preferred since they allow reducing amplification artifacts, multiplexing and successful amplification in the context of degraded DNA while producing robust and reproducible results. Finally, forensically eligible STRs are located preferably in non-coding regions on separate chromosomes or distant enough to segregate independently [26].

In general, the higher the number of analysed STR loci, the higher the discrimination power between individuals. STRs inherited independently of one another allows the application of the “product rule”, where

allele frequencies calculated through the Hardy–Weinberg equilibrium at each locus are combined across multiple genetic markers to evaluate the statistical rarity of the genetic profile and express the weight of evidence in quantitative terms [26].

To date, a common set of standardized STR markers is usually analysed for DNA testing. These STR loci were characterized and developed to address the needs of the forensic community to define a set of STR core loci for the building of forensic DNA databases, but also to share and compare genetic profiles obtained in different countries or laboratories.

In 1997, The Federal Bureau of Investigation (FBI) has selected an STR core set of 13 autosomal loci for the establishment of its database, the CODIS (Combined DNA Index System), which was extended with 7 additional loci in 2017 [33]. The other important STR marker set is the European Standard Set (ESS), established in 2001 by the DNA working group of the European Network of Forensic Science Institutes (ENFSI) and expanded in 2009 with 5 additional STRs. The extended ESS (12 STR loci in total) was formally adopted by the European Union in November 2009 to allow DNA data exchange and comparison between the European countries [26]. Nowadays a variety of commercial kits have been developed, allowing robust multiplex amplification of up to 24 STRs including *amelogenin*, a gene which allows for genetic sex identification, covering both the FBI and ESS STR core loci as well as additional STRs.

2.2.2 Single Nucleotide polymorphisms

STRs are undoubtedly the most widely used genetic markers for forensic applications because of the large amount of information collected so far in STR databases worldwide and because their analysis is based on standardized methods validated by the forensic scientific community [26]. However, other classes of genetic markers such as SNPs are receiving increasing attention since they may provide additional information for different forensic applications.

SNPs represent the most abundant class of polymorphisms in the human genome [34] and are characterized by a single-base sequence variation at a particular position in the genome that may have several alternative forms (alleles) among the individuals of a population, thus defined *sequence polymorphisms* (**Fig. 3**). The vast majority of SNPs in humans are bi-allelic markers, meaning that they may have two possible autosomal genotypes, and their average mutation rate is approximately 100 thousand times lower than that of STRs (10^{-8} versus 10^{-3}) [35]. Therefore, SNPs are not as informative as the forensically selected STRs.

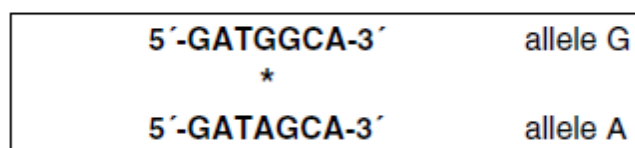


Figure 3: Single nucleotide polymorphism (SNP). The figure shows two alleles that differ at one position, indicated by the star. Figure from: *An introduction to forensic genetics* (2011) Goodwin W, Linacre A & Hadi S [30]

The 1000 Genomes Project (<https://www.internationalgenome.org/>), the SNP Consortium and the International HapMap Project have made available in the past years wide information on SNP markers and their variation in human populations. Currently, SNPs are widely analysed for association studies in medical genetics or in pharmacogenetics to identify genetic variants that influence the drug response, while in molecular anthropology, autosomal and lineage SNPs are used for reconstructing the evolutionary history of human populations and the pattern of human migrations.

Because of their low mutation rate, different classes of SNPs may be valuable for supplementary forensic analysis. Lineage SNPs located in the (non-)coding regions of the mtDNA and the Y-chromosome may be investigated in kinship analysis especially for missing person and mass disaster victims' identification where reference relatives are separated by several generations. Ancestry informative SNPs may reveal the biogeographic ancestry of an individual, aiding estimations of the biologically determined ethnic origin that may provide investigative leads to narrow the range of suspects [36]. In addition, phenotype informative SNPs are receiving considerable interest as they may allow predicting the physical appearance of a suspect, including eye, hair and skin colour, face shape and body height, assisting investigations with a more precise genetic prediction of phenotypic traits for the identification of the person of interest [37]. Identity informative SNPs have been proposed to allow genetic analyses in cases of heavily degraded DNA samples [38], thus attempt to develop identity informative SNPs panels have been reported [39, 40]. However, SNPs are significantly less polymorphic than STRs, therefore it is necessary to analyse a larger number to have the same power of discrimination of a classical composite STR profile. It has been reported that 50 SNPs need to be analysed to reach the discriminative power of 12 STR loci [41], in addition, their bi-allelic nature prevents reliable mixture interpretation [35]. Thus, despite the advantages of SNPs in the analysis of severely degraded DNA samples and the recent advances in high-throughput technologies fostering large multiplex assays, it is improbable that SNPs may substitute current STR genotyping methods.

2.2.3 Lineage Markers

Unlike autosomal DNA markers that are inherited half from the father and half from the mother, thus subjected to recombination, lineage markers are uniparentally passed down from generation to generation without changes, barring mutations. Genetic lineage markers include polymorphisms located on the mtDNA, which is transferred along maternal lineages from the mother to the offspring, and on the Y-chromosome that is inherited by paternal lineages from father to son (**Fig. 4**). Lineage markers' genetic information forms a haplotype. Haplotypes are showing only a single allele per individual and locus. Thus, considering the uniparental inheritance and the lack of recombination, such haplotypes are not as effective in differentiating between two individuals as autosomal markers are [26].

In forensic casework, a non-match between haplotypes allows excluding individuals from the range of suspects. Contrary, a match only allows identifying the maternal or paternal lineage from which the suspect with a matching haplotype cannot be excluded. The significance of a lineage marker match is decreased as

inherited as single haplotype block, thus the “product rule” applied to unlinked autosomal STR loci for the statistical evaluation of the match probability cannot be used. The current practice for the estimation of the rarity of an mtDNA or Y-chromosome haplotype among unrelated individuals involves reporting population frequencies or probabilities of haplotypes based on database searches [42]. Forensic lineage marker databases include the European DNA Profiling Group mitochondrial DNA population database (EMPOP) for mtDNA frequency estimation and the Y-STR Haplotype Reference Database (YHRD) for the Y-chromosome [26].

Although personal identification is not possible by using lineage markers, they play a valuable role in some forensic applications. The presence of even distantly related relatives having the same mtDNA or Y-chromosome haplotype allows identification of human remains in missing person and disaster victim investigations (DVI). In addition, as mentioned earlier, lineage markers are suitable to provide information about the bio-geographic origin through ancestry analyses. Forensic DNA testing for bio-geographic ancestry is useful in cases where the perpetrator is completely unknown to the investigators as such information may guide investigations and narrow the range of suspects. Lineage markers are also suitable for the reconstruction of maternal or paternal lineages, which can be useful for genealogical and historical studies as well as for tracing human migration patterns [26].

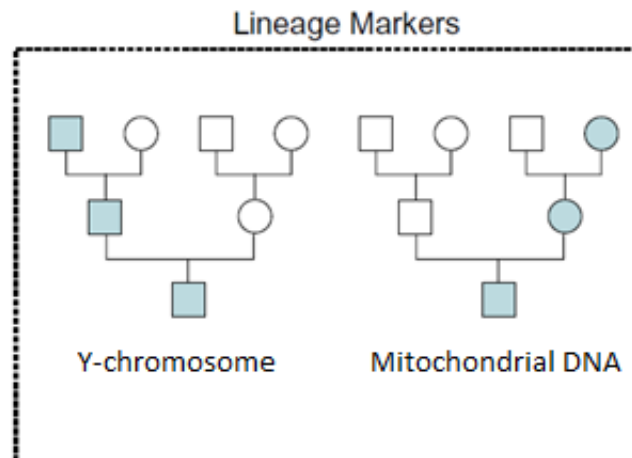


Figure 4: Lineage markers inheritance pattern. The figure shows the pattern of inheritance of Y-chromosome and mitochondrial DNA lineage markers. On the left, the paternal inheritance of Y-chromosome, transferred from father to son. On the right, the maternal inheritance of mtDNA transferred from the mother to the offspring. Figure from: *Advanced Topics in Forensic DNA testing: Methodology*, Butler (2012) [26]

2.2.3.1 Mitochondrial DNA

The mitochondrial genome is a circular double-helical molecule consisting of about 16 569 bp located within the mitochondria, the energy-producing cellular organelle, thus separated and distinct from the nuclear genome.

Today, mtDNA typing is usually performed when nuclear DNA in a sample is very limited or severely degraded and fails to produce results with standard nuclear DNA typing systems. A frequently encountered scenario with e.g. old human remains, hair shafts and teeth [43]. The likelihood of obtaining results from mtDNA analysis in such cases relies on the characteristics of this small molecule. The double membrane of mitochondria protects the mtDNA from environmental factors; moreover, its circular nature makes it less susceptible to exonucleases, thus more resistant to degradation than nuclear DNA. Finally, the presence of hundreds to thousands of copies of mtDNA in each cell increases the chances to obtain results when ancient and highly degraded or autosomal low template (LT) DNA samples are analysed [26]

The current forensic mtDNA analysis relies on Sanger sequencing of two hypervariable regions of the mtDNA non-coding control region, commonly known as HV1 and HV2. Occasionally, a third hypervariable region, HV3, may be analysed to provide additional information [43]. The obtained sequences are then compared to the revised Cambridge Reference Sequence (rCRS) [44] and results are reported in terms of variation relative to the rCRS, determining the mitochondrial haplotype of the questioned sample.

Because of the maternal inheritance, mtDNA haplotypes are grouped into broader haplogroups, which are defined by the presence or absence of typical SNPs at certain positions both of the control and the coding region of the mitochondrial genome [45]. It is assumed that all mtDNA haplotypes in the human gene pool can ultimately be traced back to a common matrilineal ancestor, thus mtDNA sequence variation, evolved as a result of the sequential accumulation of mutations along maternal lineages, can be mapped to a phylogenetic tree reflecting the relationships of known human mtDNA variation [46]. Each haplogroup appears to have characteristic geographic distribution and this would offer useful information in forensic casework, by providing ancestry information. Indeed, some lineages are associated with broad geographical regions whereas others are geographically restricted and, in some cases, even present in only one or a few ethnic groups [47], aiding to narrow the range of suspects in forensic investigations.

As previously stated, the significance of an mtDNA haplotype match in forensic cases is reduced and personal identification is not possible. In addition, Sanger-type sequencing does not allow for the ease resolution of mixtures, an endeavour which can be further complicated by the presence of heteroplasmy - a condition where more than one mtDNA type is present in a single individual [43]. However, promising new approaches involving the use of massively parallel sequencing (MPS) technologies have shown a huge variation also across the mtDNA coding region, which may assist in resolving very common haplotypes, increasing the mtDNA discriminatory power [48], and fostering deconvolution of multi-donor mixtures.

2.2.3.2 *Y-chromosome*

The human Y-chromosome is one of the smallest chromosomes found in humans, with a length of approximately 60 million nucleotides, wherein a few functional genes are present, including the *SRY* gene (sex-determining region of the Y), which determines maleness. Although the Y-chromosome pairs and

recombines during meiosis with the homologous regions of its sister sex X-chromosome at the level of two small portions located at the tips of the Y-chromosome- called pseudoautosomal regions (PAR1 and PAR2)- 95% of its sequence is not subject to inter-chromosomal recombination, thus defined non-recombining portion of the Y or NRY. The NRY is, in fact, a haploid system transmitted unchanged from father to son, barring any mutational events that may occur between generations [26].

In principle, Y-STR haplotypes are shared by all male individuals of the same paternal lineage. Therefore, forensic Y-STR typing may assist in missing person and mass disaster victim investigations as well as deficiency paternity cases and genealogy studies. In addition, by being male-specific, Y-STRs provide an additional tool to resolve cases in which multiple source cell material comes from male and female contributors, such as in sexual assault cases. On the other hand, Y-STR analysis does not allow for individualization. While exclusions can aid forensic investigations in eliminating suspects, a match only means that the individual in question could have contributed to the biological trace as any male of the same paternal lineage [49].

As with autosomal STRs, forensically suitable Y-STRs have been selected from a range of candidates and a set of STR core markers, named minimal Y-chromosome haplotype, has been proposed and chosen by the scientific community for practical use in databasing purposes and for standardization between laboratories [50]. To date, up to 27 markers are included in commercial Y-STR kits, which comprise also Y-STRs with much higher mutation rates known as rapidly mutating (RM) Y-STRs [51-52]. These RM Y-STRs are extremely useful for paternal lineage differentiation by increasing the chances of detecting Y-STR mutations that allow separating distant male relatives from close ones, which aids reducing the suspect pool [52].

Paternal bio-geographic ancestry may be inferred by analysing Y-chromosome SNPs. Similarly to mtDNA, worldwide Y-chromosome variation can be represented as a monophyletic phylogenetic tree [53] in which Y-SNPs, showing a lower mutation rate compared to STRs ($\sim 10^{-9}$ vs $\sim 10^{-3}$ per generation), are used to define Y haplogroups, hence providing useful information of the geographic origin of a Y-haplotype. In recent years, large-scale sequencing studies using MPS technologies have produced a large number of newly discovered Y-SNPs and hence Y-haplogroups, thus it is expected that further research may allow moving paternal bio-geographic ancestry inference from continental resolution to a much more detailed geographic resolution, improving criminal investigations [52].

Forensic DNA typing

3.1 Current practice in forensic genetic identification

The current practice in forensic genetic identification relies on the analysis of sets of highly polymorphic autosomal STRs markers, which are amplified by multiplex Polymerase Chain Reaction (PCR), allowing to obtain genetic profiles from minute amounts of biological material. DNA profiles obtained from evidentiary samples found at the crime scene or on evidentiary items are then compared with reference samples collected by buccal swabs or from personal objects of known individuals and, if a match is found, the statistical calculation is performed to compute the weight of the evidence. However, when the evidentiary DNA profile does not match with the reference DNA profile or with any in a forensic DNA database, this standard comparison approach cannot help in identifying individuals and solving criminal cases, but supplementary analyses as ancestry or phenotypic trait predictions may guide investigations toward the identification of a pool of individuals of interest.

3.2 Biological samples collection and pre-PCR processing

The generation of an autosomal DNA profile is an analytical process that involves multiple steps beginning with the collection of biological material, which may be a trace found at the crime scene or on evidentiary items, or cells of the oral mucosa collected through a buccal swab.

During the collection procedure, relevant biological traces are detected through an accurate visual inspection and usually collected using suitable sterile devices. DNA evidence collection must be carefully performed to avoid contamination issues. One of the most common methods for collecting cellular material is the swab technique where a sterile, moistened or dry swab is directly rubbed on the stain. However, different techniques, including scraping, tape-lifting and cutting, can be also used depending on the type of sample to be collected.

DNA can be obtained from various sources of biological material, including blood, semen, saliva, hair, sweat and skin fragments, which may be found on vaginal swabs from rape victims, underwear, chewing gum, cigarette butts, guns, clothes, and any object that may have been contacted during the criminal action. In addition, with the increase in the sensitivity of DNA profiling techniques, the recovery of DNA from epithelial cells shed on touching has also become possible [54]. Therefore, an accurate description including also photo-documentation of the evidentiary items from which the biological samples are collected is highly recommended as may guide decisions on subsequent analyses [55].

Following the sample collection, presumptive and confirmatory tests are performed to indicate whether and, if yes, which biological fluids are present. Presumptive tests, such as luminol test or forensic light sources,

are extremely sensitive but poorly specific and may be used during the collection procedure to highlight possible latent biological traces and guide their recovery. Confirmatory tests rely on the immunological reaction of specific human body fluid molecules with antibodies. These tests are highly specific and do not cross-react with other human body fluids or body fluids of other animals like some of the presumptive tests do. This allows for the identification of the biological origin of the stain, which is central for supposing the level of activity required to leave the trace [56], aiding corroborating crime circumstances. However, particularly when dealing with forensic samples, the amount of the trace may not be enough to perform both body fluid identification and DNA profiling. Recently, new molecular biology-based methods for body fluids identification have been proposed, including messenger RNA (mRNA) and micro RNA (miRNA) profiling, the analysis of the methylation pattern of tissue-specific methylation islands and microbial markers [57-60], which could help DNA analysts when classical techniques cannot be employed.

Samples showing evidence of biological material are successively subjected to DNA extraction, which allows to separate and purify the DNA molecules from other molecules. The optimal DNA extraction should, indeed, allow recovering the greater amount of DNA from forensic samples and at the same time removing any PCR inhibitors that will prevent successful profiling.

Different methods have been proposed for DNA extraction from forensic samples, including organic, chelating resin and solid-phase extraction, depending on the type of biological evidence being examined. The organic extractions involve the use of sodium dodecyl sulfate (SDS) and proteinase K to breakdown the cell membranes, next a phenol-chloroform mixture is used to separate DNA molecules from other cellular materials. Similarly, the use of chelating resins such as Chelex allows fast preparation of cellular DNA by applying a single sample manipulation step. However, over the last years, the solid phase extraction has become the most powerful technique, particularly for trace samples, designed to separate nucleic acids from the liquid phase onto a solid silica phase and later eluted in an appropriate buffer. This allows purifying the DNA extract from possible inhibitors, which may affect subsequent analyses, and reduce the risk of sample contamination or DNA loss. For reference samples commercial rapid DNA extraction methods or even direct-PCR approaches, which skip the extraction process, are currently used by many forensic laboratories [26, 30].

Once DNA in a sample has been isolated, an accurate measurement of the DNA amount and quality is desirable and strongly recommended [55] with the aim to determine the optimal amount of DNA template to produce the best quality results in terms of DNA profiling and avoid stochastic PCR effects. Indeed, a PCR amplification reaction may fail due to the presence of inhibitors, highly degraded or insufficient DNA, or a combination of all these factors. In addition, the presence of too little DNA template may result in stochastic amplification effects, as unbalanced amplification of heterozygous loci which, when severe, may determine allelic drop-out. On the contrary, the addition of too much DNA template may lead to the production of PCR artifacts as large stutter or pull-up peaks that complicate DNA profile interpretation.

Several methods may be applied to quantify the amount of DNA extract in a sample, including spectrophotometry and hybridization-based methods; however, currently, most common approaches rely on real-time PCR-based methods (qPCR). Such approaches use either SYBR Green intercalating dye, which shows a distinct increase in fluorescence upon binding to PCR products, or fluorogenic 5' nuclease assays (TaqMan), which monitor changes in fluorescence of a dual labelled probe binding within the region framed by the primers. The TaqMan probe is labelled on the 5' end with a fluorescent molecule and on the 3' end with a molecule that quenches this fluorescence. As the primers are extended by the *Taq polymerase*, the probe is degraded, releasing the fluorescent molecule and the quencher into solution, thus emitting fluorescence (**Fig. 5**). The cleavage of TaqMan probes or the binding of SYBR Green intercalating dye to double-stranded DNA molecules results in an increase in fluorescence signals which can be correlated to the DNA template amounts compared with standard DNA concentration samples, thus providing the total DNA template concentration [26].

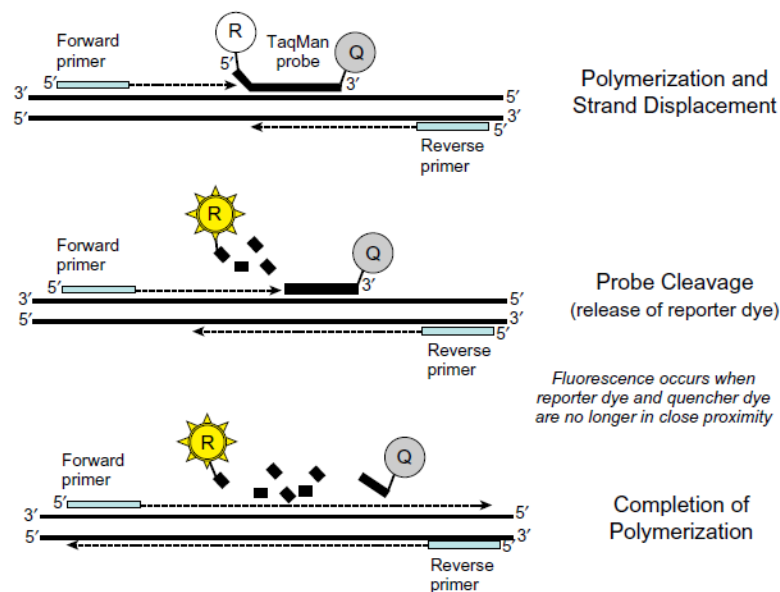


Figure 5: TaqMan qPCR assay. Figure from: *Advanced Topics in Forensic DNA testing: Methodology*, Butler (2012) [26]

Currently, several commercial qPCR kits are available for the quantification of DNA for human identity testing applications. These assays allow for the simultaneous quantification of total human DNA and male human DNA, and the detection of template degradation and PCR inhibition, thus providing an assessment of both DNA yield and purity, useful for amplification purposes.

3.3 PCR amplification and capillary electrophoresis detection

The generation of DNA profiles involves the amplification of several autosomal STR loci that are then size-separated and detected by capillary electrophoresis (CE)-based methods.

The PCR technique simulates the enzymatic DNA replication *in vitro* to amplify specific DNA regions. At present, commercial kits for identity testing allow for the simultaneous amplification of up to 23 nuclear STR loci. The amount of DNA input to be added in a PCR depends on the sensitivity of the reaction; most commercial kits are highly optimized and require between 0.5 and 2.5 ng of extracted DNA for optimal results. However, successful DNA profiling can be even performed with amounts of DNA template below 100 pg, but special attention must be paid for the interpretation of profiles becoming more complex as the amount of template DNA is reduced [61].

DNA amplification occurs in the cycling phase of PCR, which consists of three phases: denaturation, annealing and extension. In the denaturation stage, the reaction is heated to 94 °C causing the double-stranded DNA molecule to form two single-stranded molecules. As the temperature is lowered, typically between 50 and 65 °C, the oligonucleotide primers anneal to the template's complementary sequences. After the primers have annealed the temperature is increased to 72 °C to reach optimum conditions for the *Taq polymerase*, which catalyses the addition of nucleotides to the 3' ends of the primers using the original DNA strand as a template. Typically, amplification protocols utilize between 28 and 30 cycles of PCR, but in extreme cases, where the amount of DNA target is very low the cycle number can be increased to up to 34 [26]. Following the cycling phase, an incubation step between 60 and 72 °C ensures that the *Taq polymerase* adds an additional residue to the 3' end of the extended DNA molecules.

Primer pairs are designed to anneal to specific conserved regions of DNA to effectively amplify human STR loci from all populations while not binding to the DNA of other species. In order to prevent overlapping STR size ranges, one primer in every primer pair is labelled with a fluorescent dye, so that the produced PCR amplicons can be detected through their fluorescent signals when excited by appropriate wavelength light while being separated on the basis of their length during electrophoresis, thus allowing to increase the number of STR analysed simultaneously (multiplexing) (**Fig. 6**). In addition, mobility modifiers have also been applied to primers to allow shifting closely spaced STR loci, enabling an even higher level of multiplexing [26].

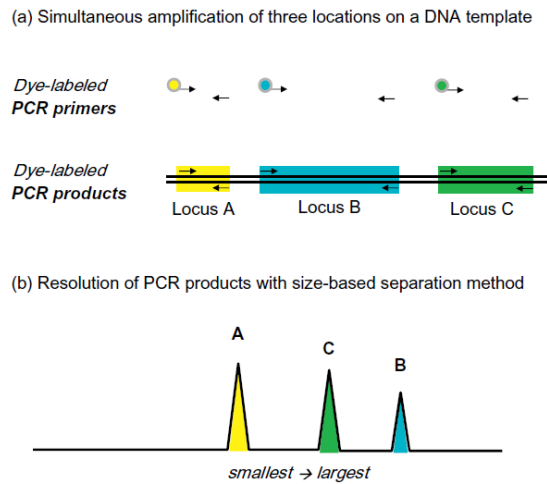


Figure 6: PCR Multiplexing. Three loci are simultaneously amplified and labelled with three different fluorochromes, thus PCR products may be separated during CE according to both their size and fluorescence. Figure from: *Advanced Topics in Forensic DNA testing: Methodology*, Butler (2012) [26]

To monitor the effectiveness of the PCR and to ensure confidence on results, controls are used, including negative controls to assess any contamination and positive controls, which consist in a standard DNA template amplified in the same batch and with the same conditions and reagents used for unknown samples [55].

Before capillary electrophoresis, PCR products are prepared by adding deionized formamide to denature DNA fragments and ensure that the amplicons are single-stranded; a fluorescently labelled internal lane size standard (ILS) is added, and a denaturation incubation at 95 °C is usually performed.

Successively, CE is used to size-separate and detect DNA fragments in the samples. Fluorescent negatively charged DNA amplicons are electrokinetically injected into a capillary containing a viscous polymer. STR amplicons migrate under the applied electric field in the capillary according to their length, which correlates with the number of base pairs, or repeats, present at a certain STR locus, the shorter the DNA molecules the faster they move, such that fragments are separated by size. As the DNA fragments migrate, a small glass window in the capillary allows an argon ion laser to excite the fluorescent dyes attached to PCR products causing an emission of fluorescence, which is detected by a charge-coupled device camera (CCD), thus allowing to further separate DNA fragments also on the basis of the colours [26]. An allelic ladder, which is an artificial mixture of the common alleles present in human populations for the analysed STRs, is added in each CE run as it allows for accurate genotype determination by providing a reference size bin for each allele included.

After data collection, the GeneMapper ID software removes spectral overlap among colour channels in the raw data and calculates the sizes of the amplified DNA fragments. To be able to size the PCR products, the instrument takes advantage of the ILS, which contains labelled DNA fragments of known lengths. These are detected along with the amplified PCR products during capillary electrophoresis, generating a size calling curve. Following the sizing, DNA fragments are then converted to STR alleles by comparing unknown

samples to the allelic ladder. After analysing the raw data, the software returns an electropherogram (**Fig. 7**) showing a series of peaks that represent different alleles. The size, peak height and peak area are also measured by the software and the amount of PCR products corresponds to the height of the peaks measured in relative fluorescent units (rfus) [26].

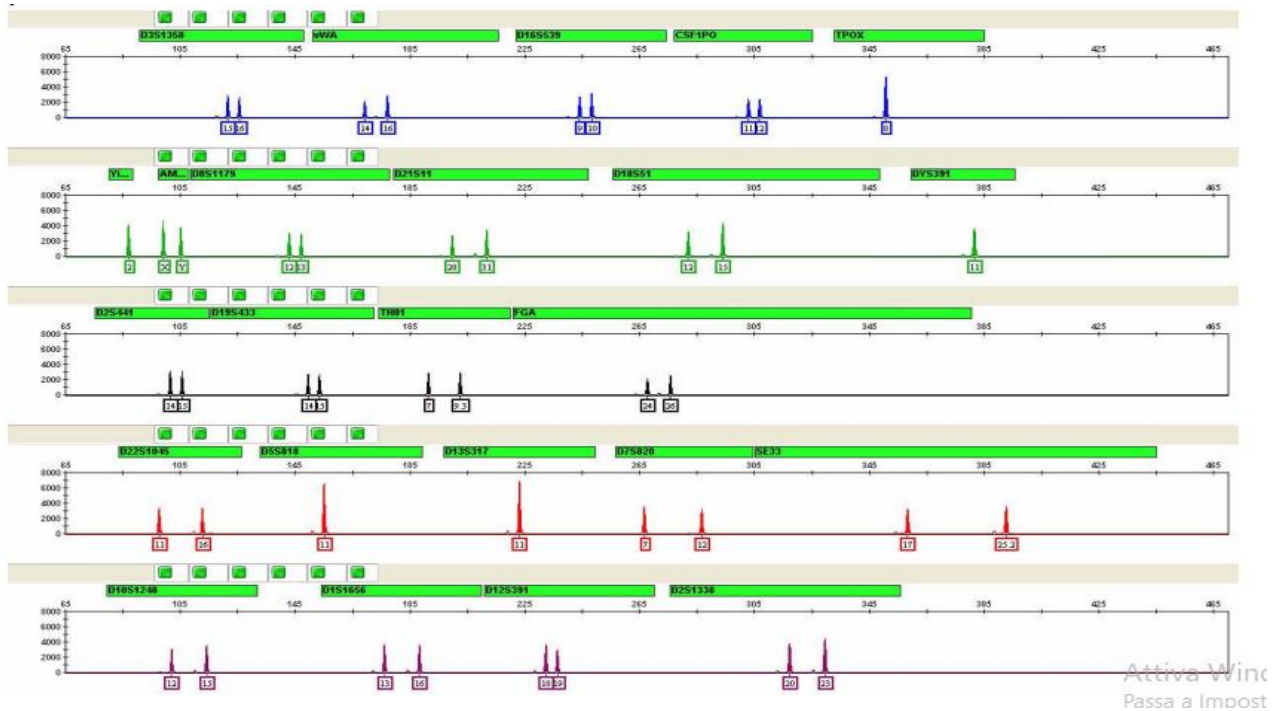


Figure 7: Electropherogram. Example of an electropherogram of DNA Control 007 amplified with the GlobalFiler kit (Thermo Fisher) and analysed on an Applied Biosystems 3500xL Genetic Analyzer. Known STR marker size ranges are highlighted with green bars, peaks represent alleles, which are named according to the number of their repetition and fall in the allelic range of the marker, rfus are reported in the left scale. Figure from: GlobalFiler™ PCR Amplification Kit USER GUIDE, available at: <http://tools.thermofisher.com/content/sfs/manuals/4477604.pdf>

3.4 Qualitative assessment and statistical interpretation of STR profiles

DNA profiles generated from forensic samples require some precautions in their interpretation to ensure that the results are robust and consistent, especially when dealing with small amounts, degraded DNA or DNA mixtures.

In conventional STR analysis, two empirical thresholds are usually set. The first one is the limit of detection or analytical threshold (AT), which allows distinguishing real peaks from background noise; it is generally set at 50 rfus and reflects the sensitivity of the CE instrument. The second, known as stochastic threshold (ST), is an interpretative threshold and defines the minimum peak height that alleles need to reach to confidentially conclude that all alleles are present at a locus [62]. The ST is usually set at 150-200 rfus and peaks above this limit are considered true alleles. If only one peak is observed at a certain locus and it is above the ST, then the locus can be designated as homozygous and the same could be done if the locus

appears heterozygous with two peaks both above the ST. On the contrary, if a single peak appears at a locus and falls below the ST then the locus is considered to be a potential heterozygote as one allele may have failed to amplify during the PCR process leading to the allele drop-out [63]. The ST may be used at data interpretation stage to assess samples in the potential danger zone of unreliable results.

While performing DNA profile interpretation, stochastic effects may be observed and should be assessed according to international scientific literature and guidelines [55, 64]. The profile interpretation involves an evaluation of the presence of PCR and CE artifacts -including stutter, spikes, pull-ups, dye-blobs and split peaks- and their impact on profile interpretation. In addition, analysts must assess with respect to the AT and ST whether or not the observed peak represents a true allele or an artifact, such as a stutter product or pull-up of signal from another dye colour. Stochastic effects, such as allelic dropout, drop-in contamination, increased intra-locus peak height imbalance, and the number of contributors need to be considered as well. DNA profiles with one or more alleles below the ST, mixed DNA profiles and degraded DNA profiles should be interpreted with caution [64]. In cases of extreme DNA profile complexity or unreproducible results, the interpretation is discouraged [55, 65].

In the condition of meaningful DNA profile, a comparison with criminal databases or reference samples from suspects or victims is performed. If the genotype comparison shows differences, the two samples have originated from different sources, thus the tested individual is excluded from the list of possible donors of the sample. Inconclusive results may be obtained in cases of extreme DNA profile complexity when there is insufficient information to support any conclusion. Finally, if the genotype comparison results in a match, i.e. the samples have the same genotypes, the weight of the evidence must be statistically calculated [26].

To date, statistical evaluation of the significance of the match may be performed according to a frequentist or a likelihood approach. The first one, known as Random Match Probability (RMP) reflects the frequency with which the particular genotype occurs in a representative population sample and illustrates the probability of another unrelated individual pulled at random from the same population having the same DNA profile. With the assumption of independence, DNA profile frequency estimates are calculated first by considering the genotype frequency for each locus using the Hardy-Weinberg equilibrium formulas and then multiplying the frequencies across all loci, according to the “product rule”. This approach may be useful for single source or single contributor profile calculations or for mixed DNA profiles clearly showing a major and a minor contributor, however, in cases of complex DNA profiles or complex DNA mixtures it is strongly recommended to use a likelihood approach, which is, in any case, the preferred method to compute the weight of DNA evidence [64 - 66]. The likelihood ratio approach (LR) relies on the comparison of the probabilities of the evidence under at least two alternative propositions reflecting different hierarchical levels that can be ultimately summarized into sub-sub source, sub-source, source, activity and offence level [66]. Propositions are mutually exclusive hypotheses and may represent the prosecution hypothesis (H_p), i.e. the DNA from the crime scene has originated from the suspect, and the defence hypothesis (H_d), i.e. the DNA has originated from an unknown person from the population coincidentally matching the defendant. LR is

calculated by dividing the hypothesis of the prosecution by the defence hypothesis and in cases of single source profiles correspond to the inverse of RMP. LR values greater than one support the prosecutor hypothesis, otherwise the defence hypothesis is supported, and verbal equivalent according to a scale of conclusions may be used to express the weight of evidence in court [67].

LR statistical calculation is required and strongly recommended in cases of mixed or LT DNA profiles as it considers alternative scenario hypotheses and permits DNA results to be combined between multiple genetic systems as well as other non-DNA evidence using Bayesian statistics [55, 64 - 67]. In addition, stutters, dropouts and drop-ins can be assessed probabilistically, thus providing a more accurate evidence evaluation. Semi-continuous biostatistics software taking into consideration drop-out and drop-in probability, as well as continuous biostatistics software that also considers peak heights or areas, stutter proportion and degradation have been developed, assisting the data interpretation and likelihood ratio calculations [68].

3.5 Challenging Forensic samples

Forensic samples collected from crime scene stains are typically far from being ideal. The biological material may have been exposed to harsh environmental conditions, ultraviolet irradiation and bacterial growth or to putrefaction fluids for days or longer times, leading to DNA samples of low quantity and low quality.

Although it is possible to obtain meaningful genetic profiles from minute amounts of DNA and different methods may improve allele detection for LT DNA samples, as increased PCR cycle number or injection time [68], the occurrence of typical LT stochastic effects and artifacts may lead to complex DNA profiles complicating the interpretation [61].

In addition to small amounts, DNA can be highly degraded, as in the case of missing person or DVI. Under the effect of environmental factors, DNA molecules are randomly broken by exonucleases and, with higher levels of degradation, more cellular material is needed to generate a DNA profile [30]. Consequently, it may not always be possible to obtain a DNA profile and, when obtained, stochastic effects may prevent its interpretation. Reduced size markers as mini-STR, designed to bind the variable repeated region as close as possible [35], or identity informative SNPs have been proposed as alternative markers to allow genotyping degraded DNA. However, with heavily degraded samples, nuclear markers may fail to give any result and the analysis of mtDNA may be the last chance (paragraph 2.2.3.1), bearing in mind that a match will identify the maternal lineage instead of the single individual.

Moreover, further complicating the interpretation of genetic results, the presence of cellular material from more than one person may lead to complex mixed DNA profiles. When male/female mixtures are observed, which are prevalent in sexual assault samples, the analysis of Y-chromosome markers may allow separating the male-specific profile from usually a high background of female DNA [50].

3.6 Sexual and physical assault investigation

Sexual and physical assaults involve direct violent contacts between a victim, often female, and an assailant, usually male, in which biological material can be transferred [21]. Therefore, victims of physical assault are

routinely subjected to detailed examination with documentation of injuries and collection of biological traces to ultimately obtain the assailant's genetic profile. DNA testing is an established and highly probative part of the investigation and prosecution of both sexual and non-sexual assaults, it allows for verifying an alleged physical or sexual contact between individuals and for challenging the victim's and the assailant's story. To date, international and national guidelines for the collection of biological evidence in sexual or physical assault investigations are available [22, 25].

Biological evidence, and particularly DNA, deteriorate with time. Therefore, it is imperative to collect sexual assault evidence as soon as possible regardless of the victim's post-assault activities, which should, in any case, be reported to allow for a proper interpretation of the genetic results. Based on the victim's history, biological stains may be collected from those areas where the perpetrator may have left his DNA. Typical sexual assault samples include swabs from the anogenital area and the oral mucosa. However, additional samples as hair, fingernail debris and trace samples collected from the skin surface or from clothes worn at the time of the crime are frequently submitted for genetic analysis particularly when no penetration or sexual attempts have been reported or if the time since the alleged assault is within the suggested detection limit [21, 22].

Case-specific information determines analysis prioritization for samples that may be most probative, and, in sexual assault cases, the presence of semen is usually investigated as it is considered the most important type of evidence to prove a sexual contact and to link the perpetrator to the victim. The serological analysis involves observation of motile spermatozoa under an optical microscope using staining such as the Kernechtrot Picroindigocarmine or Christmas Tree stain, which allows estimating the time of the assault. In addition, the observation of a reasonable number of sperm cells may direct toward a differential DNA extraction that involves a modified organic extraction method to separate the male spermatid fraction from female epithelial cells [70]. Confirmatory test allowing the identification of the semen-specific human semelogelin antigen, as RSID™ Semen, may be used for the identification of semen in dry stains.

The absence of spermatozoa or very low sperm count, such as in cases of oligospermic, azoospermic or vasectomized males, may determine a failure in the identification of a semen stain or failure to separate the male and female portions with differential DNA extraction. In addition, no ejaculation, extended time elapsed between the assault and the sampling and non-sexual physical assaults may determine a lack of semen stains, hence preventing male component separation. In such cases, other biological evidence composed by various types of non-sperm cells may still be found. However, autosomal DNA profiling can lead to complex mixtures resulting from the combination of both perpetrator and victim DNA. In such a scenario, the victim's DNA usually is in high excess, which may cause preferential amplification masking the male component, thus often precluding the identification of male contributions [71].

However, male DNA can be specifically targeted by Y-chromosome STRs analysis. Y-chromosome typing is routinely performed on sexual and physical assault samples involving female victims and male perpetrators, as it may reveal the male-specific Y-chromosome profile when no spermatozoa have been detected or when

a long time elapsed since the alleged assault [50]. In addition, the analysis of Y-STR markers in sexual and non-sexual assault cases may be central to support or improve standard autosomal STR profiling results as well as to identify multiple male contributors of a stain, determining the number of male aggressors [72].

The sensitivity of profiling techniques and the use of Y-STR typing allow for generating DNA profiles from minute amounts of male DNA in high female backgrounds that may be transferred during physical assaults against women. Since trace DNA can provide reliable results, its analysis has become an integral part in relation to both sexual and non-sexual assault investigations. However, trace DNA usually cannot be attributed to a biological source due to the small numbers of cells and the nature of transfer; therefore, to assess the biological results given the alleged activities, the significance of findings need to be based on information about factors that affect DNA transfer and persistence on the specific sampling area [56, 73]. This is particularly the case with samples collected under fingernails of women after a physical struggle in sexual and non-sexual assaults investigations.

3.6.1 Fingernail samples

In violent crimes against woman involving males as perpetrators, the occurrence of close and violent contacts leads to the transfer of biological material from the perpetrator to the victim and to the scene, and *vice versa* [21]. When the victim reports a struggle in which she might have scratched the assailant, the collection of biological material underneath fingernails may provide a useful source of DNA that may allow identifying the perpetrator [74]. The fingernail hyponychium is indeed an isolated area in which trace amounts of skin and biological fluids may accumulate, hence providing evidential material for investigation. However, when evaluating foreign DNA profiles obtained from fingernail debris, information on the prevalence, persistence and type of foreign profile obtained from fingernail samples under normal everyday *versus* crime-related circumstances need to be considered [75], particularly when the victim and perpetrator are known to each other, are in a relationship and/or live together.

Research studies have shown that the prevalence of foreign DNA under fingernails is generally low in the general population [76]. An incidence ranging from 5 to 41% of samples has been reported, however, high-quality autosomal DNA mixtures were detected in 6-14% of the cases [77-80]. When investigating factors that affected the detection of good quality mixed DNA profiles, the occurrence of recent physical or intimate contacts was found to be significant, although the nature of the contact was not further specified [78, 77], while low-level DNA profiles were associated with all levels of contact [78].

Mixed DNA profiles have been observed in 14 to 37% of the samples collected under fingernails of cohabiting individuals with 6 to 17% of them classified as high-quality [79, 81]. Notably, non-intimate cohabitation led to the exclusion of the cohabitants as the source of the foreign DNA profiles, and the time since the last contact influenced foreign profile detection [79]. Conversely, intimate cohabitants showed a higher prevalence of high-quality mixed DNA profiles compared with the general population and the amount of time a couple spent together significantly affected the detection of foreign DNA profiles [81]. Remarkably

enough, when further Y-STR profiling was performed on female fingernail samples that had previously given single donor autosomal profiles, 63% of the samples displayed full or partial Y-STR profiles [81].

Activity-related depositions of foreign DNA under fingernails were also investigated through experiments mimicking possible real criminal scenarios. A study involving vigorous scratching of male volunteers by female volunteers showed that scratching fingernails had significantly higher concentrations of male DNA, although in approximately 31% of all couples, no male DNA was detected after scratching [82]. Another scratch study revealed that immediately after scratching the scratched individual could not be excluded as the source of the foreign DNA in 33% of cases [79], however when the sampling occurred 6 hours later only 7% of samples retained foreign profiles. Similarly, a scratch experiment performed at the Legal Medicine of the University of Bologna involving females that scratched males and Y-STR typing, revealed that the scratched male could not be excluded as the source of the foreign DNA from 92.5% of the samples collected immediately after scratching and only from 25% of those collected after 5 hours [83].

Mock scenarios studies unveiled that the persistence of epithelial cells accumulated under fingernails through scratches greatly decreases over time as it is affected by normal daily activities and habits. Nevertheless, in some casework investigations, foreign DNA has been detected under fingernails also after a longer period of time and even in harsh environmental conditions [84-86]. The prevalence of foreign DNA under fingernails encountered in real forensic casework, including homicides and sexual assaults, turned out to be slightly higher than that reported for the general population; mixed DNA profiles ranged from 13 to 35% of the samples, with high-quality profiles in 7-21% of the cases [85, 79-90].

In addition, in a study on the persistence of foreign DNA after digital penetration in couples, all the samples collected from under males' fingernails immediately after digital penetration showed female source foreign profiles. However, informative profiles were still observed as mixtures in 63% of the samples collected 18 hours post penetration, revealing that the nature of the contact and thus the source of the biological material transferred highly affect both DNA transfer and persistence under fingernails [91].

Interestingly, although all these studies [79, 83, 91] involved a cleaning procedure of the fingernail hyponychium before scratching or digital penetration, foreign DNA was detected respectively in 13%, 3% and 4% of the samples collected after cleaning. The foreign profiles matched the profiles of the volunteers' real partners and some alleles could even be detected after the deliberate deposition of cellular material through the experiments. A similar outcome was found in another study involving the deposition of male sweat under female fingernail clippings where, although fingernail clippings were collected immediately after a shower in which vigorous washing with soap occurred, 19% of samples showed real partner profiles complicating the profile assessment [92].

Methods of sample recovery have an impact on the collection of the foreign material as well, and the combination of different collection methods and typing systems have been investigated. When the context

involves possible male/female mixture as fingernail samples, Y-chromosome typing has been proven to be a suitable system to specifically detect male DNA [81, 83]. In addition, regarding foreign DNA recovery, it has been shown that the collection performed finger by finger often results in very low amounts of foreign cellular material, but, as under different fingernails different contributors may be present, it may allow avoiding DNA mixtures. On the contrary, a cumulative collection performed pooling all the debris of the same hand together allows recovering higher amounts of foreign biological material, but it increases the detection of DNA mixtures [93]. Finally, sterile cotton swabs have been proven to determine higher amounts of foreign DNA recovered from under the fingernails compared to fingernail scraping and clipping [92, 93], nevertheless, a best practice consensus on the forensic analysis of fingernail samples has not emerged yet in the scientific community.

Aims of the thesis

The aim of the present thesis is to explore the mechanisms of male DNA transfer, persistence and recovery under fingernails of women following a mock physical assault as well as its prevalence during normal daily life. To achieve this a controlled scratch study was conducted involving female volunteers scratching male volunteers

The controlled scratch study may reveal information on factors that affect DNA transfer beneath fingernails under normal everyday *versus* crime-related circumstances, which may be considerably relevant in a forensic context. This holds particularly true when the victim and perpetrator are known to each other, are in a relationship and/or live together.

The possibility to distinguish between normal everyday DNA transfers and transfers due to defensive activities during a violent crime is investigated in a prevalence study, by collecting samples from volunteers two weeks before the scratch experiment. This data may serve as a baseline estimate for the background level, or prevalence, of male DNA under female volunteers' fingernails in normal daily life. Factors that may affect the detection of a foreign DNA profile under normal non-crime related circumstances, as personal habits, daily activities and human contacts in the previous 48 hours, are investigated through a questionnaire.

The level of contact required for the transfer of male cellular material beneath female fingernails under mock criminal circumstances is investigated through the controlled scratch study, involving female volunteers scratching males' forearms mimicking a defensive action during a physical assault. A cleaning procedure of the fingernail hyponychium followed by fingernail sample collection is applied to all the females immediately before scratching to reduce possible persisting genetic background deriving from previous, normal contacts of the women, for example with an intimate partner. In addition, the persistence of the exogenous cellular material is examined through targeted samplings of the fingernail debris at regular time intervals after the scratching. Variables that may affect the persistence of male DNA profiles, such as activities performed since scratching, daily habits, hygienic care and human contacts, are investigated by mean of a questionnaire administered to the volunteers following the collection of fingernail samples after scratching.

Information on variables that affect the recovery, transfer, persistence and prevalence of male DNA beneath fingernails, both under normal and criminal mock scenarios, may improve the interpretation of the significance of the genetic findings given the alleged activities in real casework and address activity level propositions. This may foster the formulation of hierarchical propositions in the LR calculation for presenting the evidence in court. Since there are so many factors that can influence the amount of foreign DNA recovered underneath fingernails, another objective of the present thesis is to compare two different sampling strategies, the cumulative and the finger by finger collection, to provide valuable insight and develop best practices for fingernail evidence analysis in forensic investigations.

Materials and methods

Scratch study design

The study protocol was approved by the Bio-ethical Committee of the University of Bologna and 48 female volunteers were enrolled for the scratch experiments.

For the prevalence study, female volunteers, after having signed the informed consent, underwent a first fingernail sample collection 14 days before the scratch experiment at the Legal Medicine of the University of Bologna. Fingernail debris collection was performed using the tipped cotton Fab-Swab 5" mini-tip (Puritan), moistened with bi-distilled water. Half of the female volunteers (24) were sampled by collecting fingernail debris cumulatively from the dominant hand, using one sterile cotton swab for all five fingernails, and finger by finger from the non-dominant hand, using one sterile cotton swab per finger, and *vice versa* for the remaining 24 volunteers. In total 6 so-called pre-experimental samples (PES) were collected per volunteer.

After the PES collection, volunteers were asked to cut or shorten their fingernails to allow nails to grow and reach an ideal length for the scratch experiment. In addition, a questionnaire (questionnaire A) was administered to each female volunteer to investigate the women's personal habits and activities performed before the PES collection. A total of 288 fingernail samples were obtained at this stage of the study and all the samples and questionnaires were anonymised using an internal laboratory female-based codification.

A buccal swab was collected from each female volunteer as a reference DNA sample using sterile cotton swabs. In addition, volunteers were instructed to collect buccal swabs from their intimate partners and cohabitants and bring swabs during the scratch days to allow the evaluation of the fingernails' genetic background.

Female volunteers were asked to return at the Legal Medicine of the University of Bologna for the scratch experiment two weeks after the collection of PES samples. For the transfer study, a cleaning procedure of the hyponychium was performed before the scratches, involving a vigorous fingernail washing with a disposable nailbrush, soap and water.

The second collection of fingernail debris was performed immediately after the cleaning procedure to reduce possible persisting genetic background, according to the cumulative/finger by finger sampling scheme used for the PES collection. A total of 288 samples were collected after fingernails cleaning and were named control samples (CS).

The scratch experiment involved the enrolment of 48 male volunteers that were asked to be present at the Legal Medicine of the University of Bologna during the scratch days, and, after having signed the informed consent, a buccal swab was collected from each of them as a reference DNA sample.

Female volunteers performed the scratches immediately after the cleaning and CS collection, by superficially scratching 10 times with both hands the forearms of male volunteers with adequate pressure, resulting in skin hyperaemia and central abrasion but without bleeding wounds.

For the transfer and persistence study, female volunteers were divided into 4 sampling groups of 12 volunteers each. The first group was sampled immediately after scratching ($t=0$), whereas the remaining groups were samples 6 hours ($t=6$), 12 hours ($t=12$) and 24 ours ($t=24$) post-scratches.

The collection of these samples was performed according to the cumulative as well as finger by finger collection scheme utilised for PES and CS. A total of 288 fingernail samples were collected after scratches and named nail samples (NS). A second questionnaire (questionnaire B) was administered to the volunteers sampled at times $t=6$, $t=12$ and $t=24$ to investigate the activity performed in the time elapsed between the scratches and the collection of fingernail samples.

DNA extraction

For all the fingernail samples ($n=864$), DNA extraction was performed at the Legal Medicine of the University of Bologna, using the silica-based QIAamp® DNA Investigator Kit (Qiagen) following the manufacturer's protocol for DNA isolation from surface swabs. A negative control (extraction blank) was included in each extraction batch and analysed alongside fingernail samples.

The swabs were placed into 1.5 mL tubes, submerged in lysis solution by adding 400 μL of Buffer ATL and 20 μL of proteinase K and incubated at 56°C for 2 hours with shaking. Tubes were briefly centrifuged and 400 μL of Buffer AL were added. After vortexing, samples were incubated at 70 °C for 10 min, then, 200 μL of absolute ethanol were added. Lysates were transferred to the QIAamp MinElute® columns and centrifuged at 8000 rpm for 1 min so that DNA molecules could bind the silica membrane. Columns were transferred into clean 2 mL collection tubes and 500 μL of Buffer AW1 were added. After centrifugation at 8000 rpm for 1 min, columns were transferred into clean 2 mL collection tubes and 700 μL of Buffer AW2 were added. Tubes were centrifuged at the same conditions and columns were transferred into clean 2 mL tubes, then 700 μL of absolute ethanol were added and tubes were centrifuged first at 8000 rpm for 1 min and, after changing collection tubes, a second centrifugation was performed at 14 000 rpm for 3 min. Columns were placed into clean 1.5 mL Eppendorf tubes and incubated at 56 °C for 3 min with lids opened. DNAs were eluted by adding 50 μL of Buffer ATE to the centre of the membranes. An incubation step at room temperature for 5 min was performed before the final DNA elution to increase DNA yield, then tubes were centrifuged at 14 000 rpm for 1 min to elute DNA.

All the fingernail DNA extracts and reference buccal swabs were brought to the Institute of Legal Medicine of the Medical University of Innsbruck (AT) for subsequent genetic analyses.

Buccal swabs DNA extraction was performed at the Institute of Legal Medicine of the Medical University of Innsbruck, by using a rapid Chelex based extraction method [94]. 10 g of Chelex 100 resin® (BioRad) were suspended in 100 mL of HPLC grade deionized water and 8 mL of Proteinase K (10mg/mL) were added. Buccal swabs were placed into 1.5 mL Eppendorf tubes, Chelex suspension was placed on a magnetic stirrer and 1250 µL of suspension were added to each tube. Tubes were placed into a thermomixer (Eppendorf) and incubated first at 56 °C for 50 min with 800 rpm shaking, then at 95 °C for 15 min without shaking. Samples were cooled down to room temperature, vortexed and centrifuged for 5 min at 4500 rpm.

All fingernail and reference DNA extracts were stored at -20 °C until further use.

DNA quantification

Fingernail DNA extracts and extraction blanks were quantified for the presence of male total genomic DNA. The quantification of male DNA was performed in duplicate for PES and CS and singularly for NS.

Male DNA quantification was performed by means of a real-time PCR quantification assay [95] targeting a multi-copy sequence located on the Y-chromosome, known as Y-chromosome repetitive sequence (YRS). The YRS qPCR assay has been carried out using the ABI Prism 7500 instrument using the 7500 Software v2.3 (Thermo Fisher). All reactions were carried out in a final volume of 10 µL using 2 µL of the sample, 1X TaqMan Advanced Fast MasterMix (Thermo Fisher) and non-acetylated bovine serum albumin (BSA) at 0.125 µg/µL concentration. Sequences of qPCR primers (YRS-F and YRS-R) and the TaqMan probe (YRS-P) were taken from the literature [95] and primers and probe sequences and final concentrations in the quantification reaction are listed in Table 1. qPCR conditions were as follows: 95 °C for 20 s, 40 cycles at 95 °C for 3 s and 60 °C for 30 s.

Sensitivity and specificity of the reaction were tested on standard dilutions of male DNA positive control and a female no-amplification control DNA preparation.

Table 1: YRS quantification assay. Primers and probes description and final concentrations for 10 µL volume reaction [95]

Primers and probe	Final Concentration	Sequences
YRS-F	300 nM	5'- GATGAGTGTTACAGCACTTAAAGGTGT -3'
YRS-R	300 nM	5'- GATGAGGTCTGCAGCTTCATTCT -3'
YRS-P	200 nM	5'- 6FAM-TCTTGCTCACTTCAA-MGB/NFQ-3'

For all the NS (n=288) autosomal DNA was quantified according to the SD quant protocol [96]. The quantification assay relies on tetraplex real-time PCR quantification which allows the simultaneous quantification of autosomal and mtDNA by targeting a multi-copy nuclear DNA region and two different-sized mtDNA targets, including an internal positive control (IPC) to monitor potential inhibition. All

reactions were carried out in a final volume of 20 μ L using 2 μ L of the sample, 1X TaqMan Advanced Fast MasterMix (Thermo Fisher) and non-acetylated bovine serum albumin (BSA) at 0.125 μ g/ μ L concentration. Primers and probe sequences and their final concentrations in the multiplex are listed in Table 2. Amplification and fluorescence detection were performed using an ABI Prism 7500 instrument using the 7500 Software v2.3 (Thermo Fisher). qPCR conditions were: 95 °C for 20 s, 40 cycles at 95 °C for 3 s and 60 °C for 30 s.

Table 2: SD quant assay. Primers and probes description and final concentrations for 20 μ L volume reaction [96].

Probes and primers	Final Concentration	Sequences
nRNU2-F	900 nM	5'-GGA TTT TTG GAG CAG GGA GA-3'
nRNU2-R	900 nM	5'-CTG CAA TAC CAG GTC GAT GC-3
RNU probe	500 nM	5'-FAM GAG CTT GCT CCG TCC ACT CC-MGB/NFQ
mtND1-F	300 nM	5'-CCC TAA AAC CCG CCA CAT CT-3'
mtND1-R	300 nM	5'-GAG CGA TGG TGA GAG CTA AGG T-3'
mtND1 probe	100 nM	5'-VIC CCA TCA CCC TCT ACA TC-MGB/NFQ
mt143bp_F	300 nM	5'-CCA CTG TAA AGC TAA CTT AGC ATT AAC C-3'
mt143bp_R	300 nM	5'-GTG ATG AGG AAT AGT GTA AGG AGT ATG G-3'
mt143bp probe	100 nM	5'-NED CCA ACA CCT CTT TAC AGT GAA-MGB/NFQ
IPC-F	100 nM	5'-ATC AGC TTA GCG TGC AGT CA-3'
IPC-R	100 nM	5'-TCT TCG TCG TAA CGG TGA GC-3'
IPC-probe	100 nM	5'-Cy5 GTT GCA CTA CTT CAG CGT CCC A-3'
IPC-oligo	0.001 nM	5'-ATC AGC TTA GCG TGC AGT CAG ATA ATG TTG CAC TAC TTC AGC GTC CCA AGC TCA CCG TTA CGA CGA AGAG-3'

Nail samples (NS) PCR amplification

All samples collected after scratching (NS, n=288) were analysed for Y- STRs typing.

Y-STR amplifications were performed using the PowerPlex® Y23 System (Promega Corporation) that allows co-amplification and four-colour fluorescent detection of 23 Y-chromosomal loci (**Fig. 8**), including DYS576, DYS389I, DYS448, DYS389II, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438, DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643, DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4. All necessary materials to perform Y-STR typing are provided by the kit, including hot-start thermostable DNA polymerase, buffer, MgCl₂ and dNTPs, which are components of the PowerPlex® (PP-)Y23 5X Master Mix. Primer pairs are included in the Primer Pair Mix and 2800M Control DNA, amplification grade water, PP-Y23 Allelic Ladder Mix and Internal Lane Standard WEN 500 PP-Y23 are also provided with the kit.

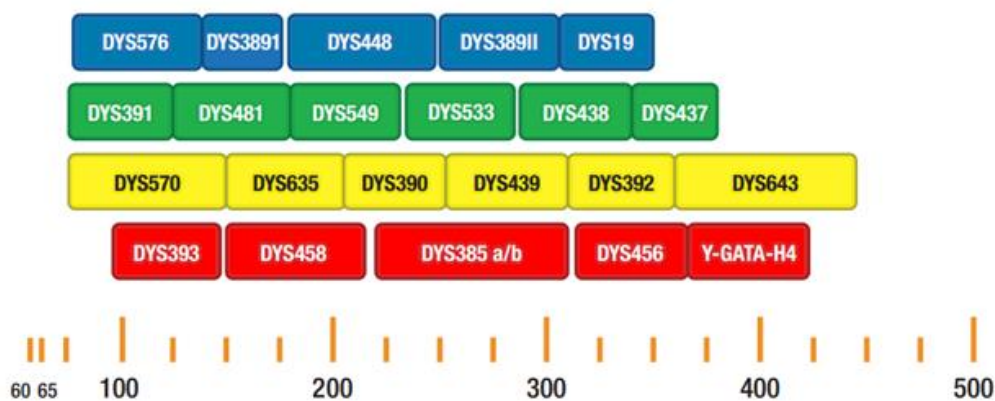


Figure 8: PCR product size ranges and dye colour configurations for STR loci present in PowerPlex® Y23 System®. The ILS WEN 500 is represented in the orange channel. Figure from: Technical manual in PowerPlex® Y23 System for Use on the Applied Biosystems® Genetic Analyzers, available at <https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/powerplex-y23-system-protocol.pdf>

PP-Y23 amplifications were performed according to the manufacturer's protocol for extracted DNA, by adding 5 µL of PP-Y23 5X Master Mix, 2.5 µL of 10X Primer Pair Mix, 500 pg of DNA template whenever possible, and nuclease-free water to a final volume of 25 µL. For samples failing to reach the optimal DNA input of 500 pg, the maximum possible volume of 10 µL was added.

PP-Y23 PCR reactions were carried out on a 9700 GeneAmp® PCR System (Applied Biosystems) and PCR cycling conditions were as follows: 96 °C for 2 min, 30 cycles at 94 °C for 10 s, 61 °C for 1 min and 72 °C for 30 s, followed by a final incubation at 60 °C for 20 min.

NS showing useful PP-Y23 profiles were also analysed for autosomal STRs.

Autosomal DNA amplification was performed using the PowerPlex® ESX17 System (Promega Corporation), which allows co-amplification and four-colour fluorescent detection of 16 autosomal STR loci and amelogenin (**Fig. 9**), including D18S51, D21S11, TH01, D3S1358, D16S539, D2S1338, D1S1656, D10S1248, FGA, D8S1179, vWA, D22S1045, SE33, D19S433, D12S391 and D2S441. The PowerPlex® ESX17 System allows amplification of the loci recommended by the European Network of Forensic Science Institutes (ENFSI) and European DNA Profiling Group (EDNAP) as mini-STRs (D2S441, D10S1248 and D22S1045, with length lower than 125bp;) and midi-STRs (D1S1656 and D12S391, with lengths ranging from 125 to 185bp). The kit provides all materials necessary to perform DNA amplification, including hot-start Taq DNA polymerase, as components of the ESX 5X Master Mix, while primer pairs are included in the Primer Pair Mix. Positive 2800M Control DNA, amplification grade water, ESX17 Allelic Ladder Mix and Internal Lane Standard WEN ILS 500 are also provided with the kit.

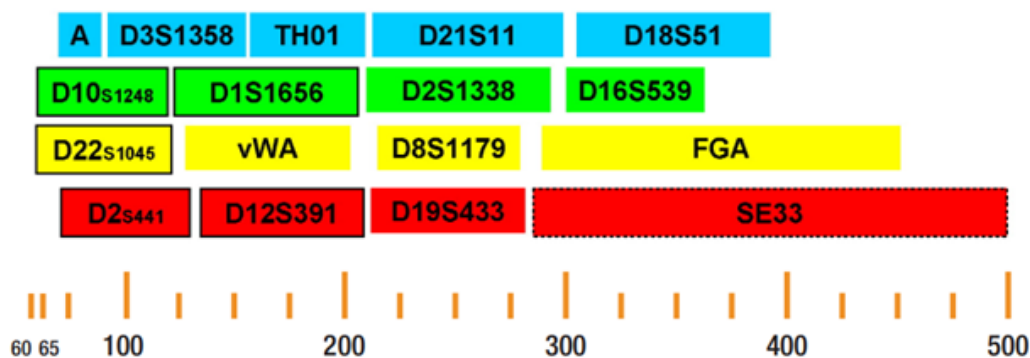


Figure 9: PCR product size ranges and dye colour configurations for the STR loci present in PowerPlex® ESX17. The “A” in the blue channel is amelogenin marker. The ILS 500 is represented in the orange channel. Figure from: *Concordance and population studies along with stutter and peak height ratio analysis for the PowerPlex® ESX17 and ESI 17 Systems.* (2011) Hill, Carolyn R. et al. *Forensic Sci Int Genet*, 5(4), 269–275. doi: <https://doi.org/10.1016/j.fsigen.2010.03.014>

PowerPlex (PP-)ESX17 PCR were optimized in half of the volume recommended by the manufacturer and the amount of DNA template input was determined through an internal validation study; the final volumes of all reagents are listed in Table 3.

Table 3: PowerPlex® ESX17 PCR Amplification Mix for 12.5 µL volume reaction.

PP-ESX17 Master Mix	Input
Nuclease-free water	to a final volume of 12.5 µL
5x Master Mix	2.5 µL
10x Primer Pair Mix	1.25 µL
DNA template	130 pg

PP-ESX17 PCR amplifications were carried out on a 9700 GeneAmp® PCR System (Applied Biosystems) and PCR cycling conditions were as follows: 96 °C for 2 min, 30 cycles at 94 °C for 20 s, 59 °C for 2 min, 72 °C for 30 s, followed by an extra extension step at 72 °C for 5 min and final incubation at 60 °C for 10 min.

A positive and a negative PCR control were included in each amplification batch, both for PP-Y23 and PP-ESX17, and extraction blanks were amplified together with fingernail samples.

PCR amplification of reference samples

All the DNA extracts obtained from reference buccal swabs were amplified using the same kits and the same analysis conditions used for NS.

Pre-experimental samples (PES) PP-Y23 profiling

On the basis of Y-STR results obtained for NS, logistic regression was performed to define a Y-chromosome quantification cut-off for PES Y-STR typing. The model was constructed on 75% and tested on 25% of the entire NS dataset using base R routines [97].

PP-Y23 amplification for PES was performed at the same condition used for NS only for those PE samples that showed amounts of male DNA above the Y-chromosome quantification cut-off.

Capillary electrophoresis detection

PP-Y23 and PP-ESX17 products were separated by capillary electrophoresis using the Biosystems® 3500xL Genetic Analyzer (Thermo Fisher). The automated pipetting system Eppendorf epMotion P5073 (Eppendorf) was used to set up 96-well plates for capillary electrophoresis runs, by adding for each sample 9.5 µL of Hi-Di Formamide™ (Thermo Fisher) and 0.5 µL of either WEN ILS 500 Y23 for PP-Y23 profiling or WEN ILS 500 for PP-ESX17 profiling. 1 µL of the amplified sample was added to the mix and PP-Y23 or PP-ESX17 Allelic Ladder Mix was included in each CE run as well as a 2800M control DNA and a negative CE control.

Data collection software was used to collect raw data and GeneMapper IDX v 1.2 (Thermo Fisher) was used for allele calls and profile analysis.

PPY23 profiles interpretation

Based on internal validation studies, a minimum peak height threshold of 50 rfus was used for the blue channel, 80 rfus for the green and 100 for the yellow and red channels [98]. Peaks being one repeat unit shorter and lower than 15% of the main peak height were considered stutter peaks.

PP-Y23 profiles were defined full or partial according to the number of amplified Y-STRs and partial Y-chromosome profiles with less than 10 amplified loci were considered not useful for comparison purposes due to that high risk of coincidental haplotype matches [99]. Single source PP-Y23 profiles (SS), either full or partial, were characterized by one allele at each single-copy locus, while the presence of two or more alleles at more than 4 single-copy loci was used to identify a mixture (MIX). Full or partial Y-STR profiles with more than one allele at 1-4 loci were defined drop-in profiles (DI).

To give a perspective on the quality of male DNA profiles, an interpretative threshold of 300 rfus was used and when all the peaks of full Y-STR profiles, excluding clear drop-in alleles, reached the interpretative threshold, Y-STR profiles were defined reportable (REP), while full or partial Y-STR profiles with no more than 4 allelic drop-outs were defined suitable (SUIT). Partial PP-Y23 profiles with more than 4 allelic dropouts and/or with peak height average lower than 250 rfus, showing clear presence of stochastic effects, were defined low template (LT).

PP-Y23 profiles obtained from fingernail samples were compared to the relevant male participants' reference samples and likelihood ratios were calculated for those Y haplotypes matching the person of interest, i.e. the scratched male or volunteers' partner and male cohabitants, considering that Y-haplotype LR calculations are based on the frequency of the questioned haplotype in the reference population. Y-haplotype frequencies were determined through YHRD searches (www.yhrd.org) [100]. The YHRD release 61 contains more than 285 000 minimal Y haplotypes from more than 1000 worldwide populations. Yet, the database searches performed were restricted to European populations only.

For single source and drop-in Y-STR profiles, haplotype frequency estimation was carried out using the counting method when the haplotype was already seen in the database (not considering mixed loci) and the LR was calculated as the inverse of the haplotype frequency [101]. For unobserved haplotypes, a reduced locus-count search was first performed followed by the counting method estimation or using the κ correction method for unobserved haplotypes [102]. For mixed Y-STR profiles, the LR was calculated by YHRD's Mixture Analysis tool [103].

PP-ESX17 profiles interpretation

Analytical thresholds were based on internal validation studies and a minimum peak height threshold of 50 rfu was used in the blue channel, 80 rfu in the green, and 100 rfu in the yellow and red channels [98]. Peaks one repeat unit shorter and lower than 15% of the main peak height were considered stutter peaks.

As with Y-STR profiles, PP-ESX17 profiles were defined full or partial according to the number of amplified STR. Single source PP-ESX17 profiles (SS), either full or partial, were characterized by no more than two alleles at each locus, while the presence of more than two alleles at more than 4 loci was used to identify a mixture (MIX). Full or partial PP-ESX17 profiles with more than two alleles at 1-4 loci were defined drop-in profiles (DI).

The weight of evidence was assessed by comparing fingernail and references profiles. CaseSolver software [104] (available at <http://www.euroformix.com/casesolver>) was used for the automatic calculation of LRs, but when the software failed to detect a contribution, LRs were calculated using EuroForMix package [105] directly from the CaseSolver interface.

Statistical analyses

All statistical analyses were carried out using R software v. 3.6.1 [97].

R software was used to run CaseSolver and EuroForMix packages, as well as for statistical analyses and figures, using the package ggplot2 [106].

Statistical significance was attributed at the 5% type I error level and to correct for multiple hypotheses, Bonferroni correction was applied.

Results

Male gDNA quantification results

The YRS qPCR assay resulted in a sensitivity ranging from 20 ng/ μ L down to less than 1 pg/ μ L of male DNA (**Fig. 10**) and the specificity of the quantification reaction was demonstrated by no amplification of the female control DNA.

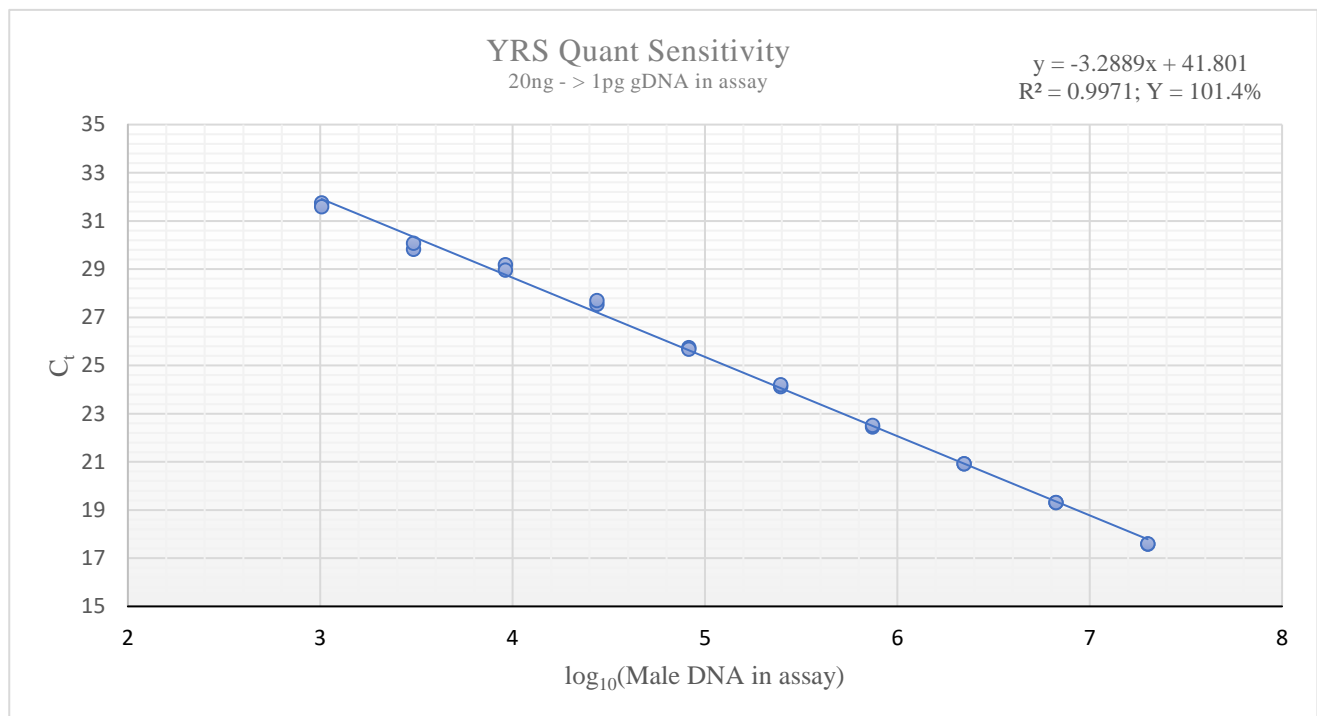


Figure 10: Sensitivity of YRS qPCR assay. Standard curve plot of the quantification range. Amounts of male DNA (fg/ μ L) are reported on the x axis in \log_{10} scale, and cycle threshold (C_t) values are reported on the y axis. Regression line parameters are also reported.

Male DNA quantification data for NS ranged from nominally 0 pg/ μ L to approx. 10 ng/ μ L and, on 288 NS, 14 failed to produce any quantification result. Autosomal targeted based DNA quantification values ranged from approx. 1 pg/ μ L to 10.4 ng/ μ L.

PES showed amounts of male DNA ranging from nominally 0 to 46 pg/ μ L and CS from 0 to 13.2 pg/ μ L, with 30 samples failing to produce Y-chromosome quantification data.

Descriptive statistics for male-specific YRS-based DNA quantification result of PES, CS and NS sampling groups are summarized in Table 4.

Table 4: Descriptive statistics of male DNA quantification values (pg/ μ L) for PES, CS and NS sampling groups (SG). Mean, standard deviation, coefficient of variance minimum, first quantile, median, third quantile, maximum, interquartile range, mean absolute deviation, kurtosis and skewness values are reported.

SG	mean	Sdev	CV%	min	Q1	med	Q3	max	IQR	MAD	kurt	skew
PES	2.1	5.4	258.7	0.0	0.2	0.6	1.5	46.0	1.2	0.6	36.1	5.6
CS	0.4	1.2	313.5	0.0	0.0	0.1	0.2	13.2	0.2	0.1	56.7	6.8
NS	51.2	620.7	1212.7	0.0	0.1	0.6	3.1	10384.5	2.9	0.9	272.3	16.3

Pairwise comparisons using the Wilcoxon rank sum test for non-normally distributed data showed a statistically significant difference in male DNA amounts for CS sampling group, both against PES and NS sampling groups (p-value= 2 e-16). A graphical representation of Y-chromosome DNA quantification results obtained in the three sampling groups, PES, CS and NS, is given in Figure 11.

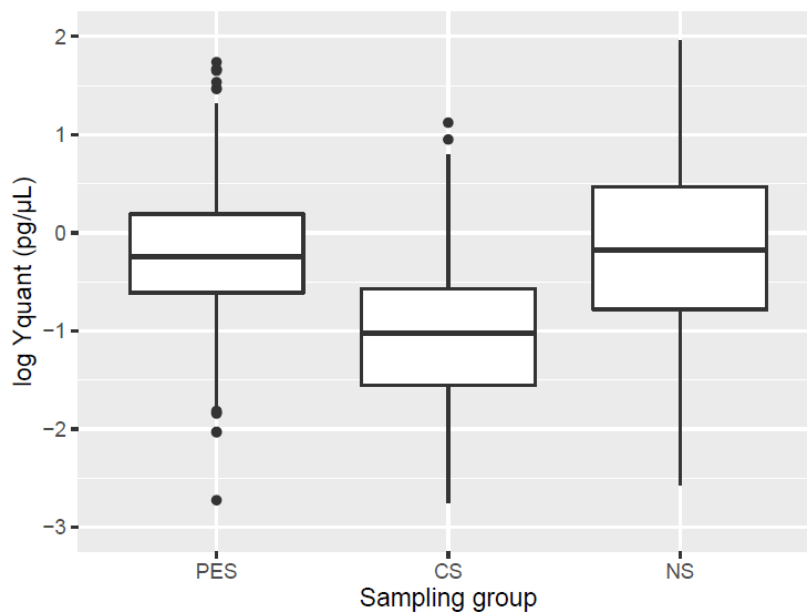


Figure 11: Box-whiskers plot of male DNA quantification data obtained for PES, CS and NS. Male DNA quantification results (pg/ μ L) are plotted in log10 scale on the y axis and extreme values are removed. The different sampling groups (PES, NS and CS) are reported on the x axis.

Statistically significant differences in male DNA amounts were found also in NS sampling group with respect to the different collection times. Specifically, t=0 samples collected immediately after scratches displayed significantly higher quantification values of male DNA than t=6 (p-value= 6.6 e-11), t=12 (p-value=2.2 e-10) and t=24 samples (p-value= 1.5e-8). Descriptive statistics for Y-chromosome quantification result for the different NS collection times, t=0 t=6, t=12 and t=24, are reported in Table 5 and a graphical visualization is given in Figure 12.

Table 5: Descriptive statistics of male DNA quantification values (pg/ μ L) for $t=0$, $t=6$, $t=12$ and $t=24$ hours post-scratching NS collection times. Mean, standard deviation, coefficient of variance, minimum, first quantile, median, third quantile, maximum, interquartile range, mean absolute deviation, kurtosis and skewness values are reported.

Collection time	n	mean	Sdev	CV%	min	Q1	med	Q3	max	IQR	MAD	kurt	skew
$t=0$	72.0	199.5	1240.4	621.8	0.0	1.0	3.4	13.3	10384.5	12.3	3.9	67.6	8.1
$t=6$	72.0	1.3	2.5	188.5	0.0	0.1	0.3	1.1	11.5	1.0	0.5	8.2	2.9
$t=12$	72.0	2.8	9.2	329.5	0.0	0.1	0.3	1.0	51.2	0.9	0.4	21.8	4.7
$t=24$	72.0	2.5	5.6	222.9	0.0	0.0	0.3	2.1	35.5	2.0	0.5	17.8	3.8

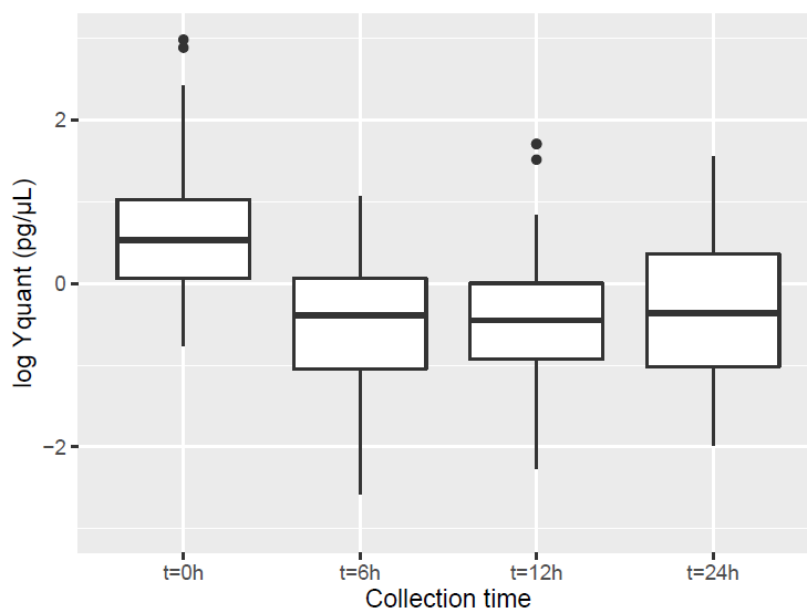


Figure 12: Box-whiskers plot of male DNA quantification data for $t=0$, $t=6$, $t=12$ and $t=24$ NS collection times. Male DNA quantification results (pg/ μ L) are plotted in \log_{10} scale on the y axis and extreme Y-chromosome quantification values are removed. The different collection times ($t=0$, $t=6$, $t=12$ and $t=24$) are reported on the x axis.

Male DNA recovery study results

Concerning the two different sampling strategies applied to the female volunteers for the collection of fingernail samples, pairwise comparisons using the Wilcoxon rank sum test showed no statistically significant differences in YRS-based quantification values between the dominant and non-dominant hands both when quantification data were tested altogether and when tested within the PES, CS and NS sampling groups.

The cumulative collection method produced on average higher male DNA yields than the finger by finger approach. Furthermore, Wilcoxon rank sum test revealed statistically significant differences in the cumulative collection, resulting in higher male DNA amounts than the 5 fingers, with p-values of 2.2×10^{-5}

for finger 1, 2.3×10^{-4} for finger 2, 5.5×10^{-4} for finger 3, 8.0×10^{-6} for finger 4 and 3.4×10^{-8} for finger 5. No statistically significant differences were found among the individual fingers.

Within sampling groups, Wilcoxon test revealed statistically significant differences in the PES sampling group for the cumulative collection, resulting in higher male DNA amounts than all the 5 fingers, with p-values of 5.1×10^{-6} for finger 1, 1.1×10^{-2} for finger 2, 1.6×10^{-3} for finger 3, 5.9×10^{-6} for finger 4 and 3.4×10^{-7} for finger 5. In addition, for quantification results of the dominant hands no statistically significant differences were found for the cumulative collection against the 5 fingers, while significant differences were found for the non-dominant hand, where the cumulative collection yielded significantly higher male DNA amounts than all the 5 fingers, with p-values of 4.0×10^{-6} for finger 1, 7.7×10^{-4} for finger 2, 9.6×10^{-4} for finger 3, 2.2×10^{-5} for finger 4 and 5.8×10^{-6} for finger 5 (**Fig. 13**).

Across the CS sampling group, Wilcoxon test showed statistically significant differences in male DNA recovery between the cumulative collection against the 5 fingers only for finger 4 (p-value=0.01) and 5 (p-value=0.031). No statistically significant differences were found among male DNA amounts for the cumulative collection against the 5 fingers from the dominant hand, while significant differences were found for the non-dominant hand, where the cumulative collection resulted in significantly higher DNA amounts than finger 4 (p-value=0.015) and finger 5 (p-value=0.022) (**Fig. 13**).

Within the NS sampling group, statistically significant differences in male DNA recovery were revealed by Wilcoxon test between the cumulative collection against the 5 fingers for finger 1 (p-value=0.003), finger 2 (p-value=0.037) and finger 5 (p-value=0.0072). No statistically differences were found among male DNA amounts for the cumulative collection against the 5 fingers from the dominant hand, while significant differences were found for the non-dominant hand, where cumulative collection yielded significantly higher DNA amounts than finger 1 (p-value=0.0132) (**Fig. 13**).

The comparisons between cumulative vs finger by finger collection methods of the dominant and non-dominant hand among PES, CS and NS sampling groups and within each sampling group, is depicted in Figure 13.

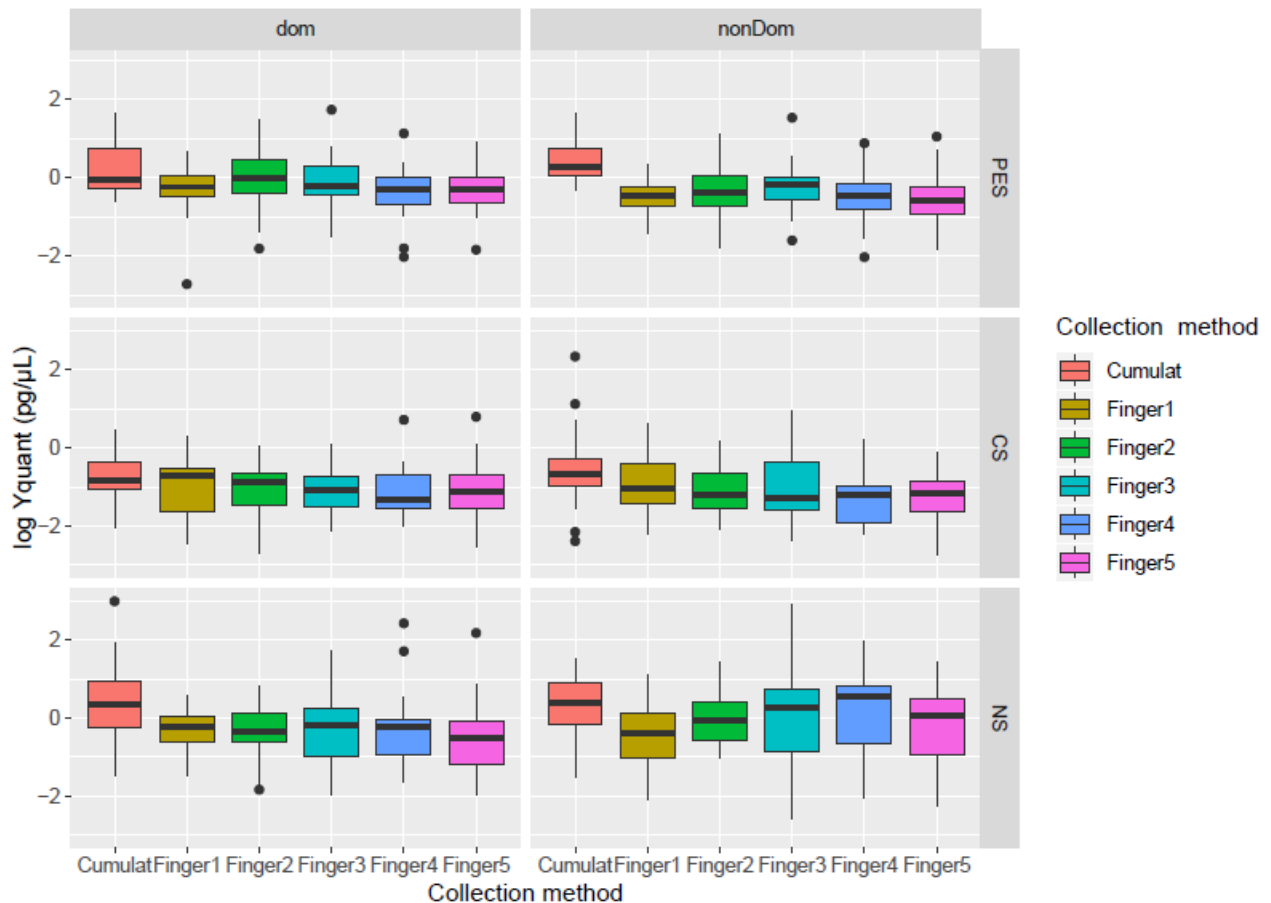


Figure 13: Box-whiskers plot of male DNA quantification values with regard to the collection method and hand dominance. Male DNA quantification values (pg/μL) are reported on the y axis in log₁₀ scale and extreme values are eliminated to allow a better visualization of boxplots. Cumulative vs finger by finger collection methods are reported on the x axis. The comparison of collection methods for the dominant and non-dominant hand is reported both among and within sampling groups (PES, CS and NS).

Male DNA prevalence study results

Logistic regression was performed on a training set comprising 75% of all NS (n = 288) with known male DNA content and PP-Y23 profiling classification (for details see Tables 7-10). A probability of 14.2% for yielding a useful PP-Y23 profile was used as threshold setting and tested on the remaining 72 NS forming the test set for predicting PP-Y23 typing success on the basis of male DNA content. This value was found to be the highest setting resulting in zero false-negative predictions while producing only 5.6% false positives with the NS set. The specificity of the model was found to be 91.3% and the overall accuracy amounted to 94.4% (95% CI: 86.2–98.4%) with an “area under the curve” (AUC) metric of 98.3%, underlining the high predictive power of male DNA content for PP-Y23 genotyping success. The 14.2% success probability threshold translated into a male DNA content cut-off of approximately 0.8 pg/μL, which was applied for PES Y-STR typing.

For the prevalence study of male DNA under volunteers' fingernails, among 288 PES collected 14 days before the actual scratch experiment, a total of 110 DNA extracts (38.2%) showed amounts of male DNA higher than the defined cut-off of 0.8 pg/ μ L and PP-Y-23 amplification was performed on this subset of PES. The remaining 178 (61.8%) samples contained insufficient or undetectable amounts of male DNA to proceed with Y-STR typing. Among the 110 samples with adequate male DNA amounts, 2 were found to be the result of a contamination event, hence, excluded from statistical calculations. Consequently, the effective count of PES with male DNA contents exceeding the pre-defined 0.8 pg/ μ L threshold amounted to 108 (37.5%). Among these, 57 samples resulted in PP-Y23 profiles with less than 10 Y-STRs amplified, thus defined not useful, while 51 gave useful Y-STR profiles with 10 or more Y-STRs amplified, corresponding to 17.7% of the entire PES set.

The quality of Y-STR profiles ranged from reportable full single source or drop-in profiles, obtained in 11.8% of the successfully analysed samples (n=6), to partial low template Y-chromosome profiles representing approximately 60.8% (n=31) of the PES giving useful Y-chromosome profiles. Suitable Y-STR profiles were found both among full and partial PP-Y23 profiles, whether drop-in, single source and mixed, for a total of 14 PES (27.4%) (**Tab. 6**).

Female volunteers were instructed to provide reference buccal samples from their partner and cohabitants, resulting in 24 male partner and 42 cohabitant reference samples. Thirteen of these cohabitants were males.

The comparison of PES PP-Y23 profiles with reference profiles from female volunteers' partners and male cohabitants resulted in 29 profiles being compatible with the partners' reference sample profiles. These 29 samples represented 56.9% of all successfully typed PES or 10.1% of the entire PES set. Noteworthy, 5 of these could also have derived from female participants' sons.

Ten of the 29 profiles were classified suitable (full single source and drop-in) and 14 were classified as low template partial profiles.

Y haplotypes not matching any reference samples were found in 22 cases (43.1%), including 1 reportable full drop-in profile, 4 suitable partial drop-in and mixed profiles and 17 low template partial profiles. Their share on the entire PES set was 7.6%. Six of the unknown haplotypes, being either classified as suitable partial mixed (n=1) or as partial low template (n=5), showed one or two incompatibilities with the relevant reference Y haplotypes.

Table 6 summarizes PES PP-Y23 typing results, including Y-STR profile classification and quality, the number of loci detected and the number of loci compatible with volunteers' partners or cohabitants. The quantity of male DNA (pg/ μ L) and comparison results are also reported.

Table 6: PES PP-Y23 typing results for the PES producing useful profiles with 10 or more Y-STRs amplified. PP-Y23 profiles classification includes FULL and PARTIAL single source (SS), drop-in (DI) and mixed (MIX) Y-STR profiles. PP-Y23 profiles quality, comprising low template (LT), suitable (SUIT) and reportable (REP) categories, the amount of male DNA (pg/μL) per sample, the number of Y-STR loci detected and the number of compatible Y-STR loci with respect to male partner (MP) and male cohabitants (MC) reference Y-STR profiles are reported. For the duplicated locus DYS385 a score of 0.5 was assigned per each compatible allele. The match with reference profiles is indicated as MS, MP and MC and unknown Y haplotypes are termed “U”. “MP/S” is used when volunteers’ son’s reference Y haplotype was available. Y-STR profiles showing one or two incompatibilities with the reference Y haplotypes are indicated with “/” in the match column.

Sample	Y-quant (pg/μL)	PP-Y23Profile Classification	PP-Y23Profile Quality	Detected Loci	Compatible with		Match
					MP	MC	
F1_PES_C	2.17	PART DI	LT	10	1		U
F7_PES_5	7.97	PART SS	LT	15			U
F9_PES_C	6.05	PART MIX	LT	13			U
F9_PES_2	4.75	PART SS	LT	12			U
F9_PES_3	2.38	PART DI	LT	14			U
F11_PES_C	5.21	PART DI	LT	12	12		MP
F11_PES_1	2.00	PART DI	LT	11	10		/
F11_PES_3	6.04	PART SS	LT	13	12		/
F14_PES_C	44.65	FULL DI	REP	22			U
F15_PES_5	3.61	PART DI	LT	16	5		U
F17_PES_C	8.33	PART SS	LT	11			U
F17_PES_4	4.92	PART DI	LT	16			U
F20_PES_2	12.59	PART DI	SUIT	21	8		U
F21_PES_C	20.35	PART DI	SUIT	19	19		MP
F23_PES_C	29.19	FULL MIX	SUIT	22	22	12	MP
F23_PES_1	4.71	PART DI	LT	10	10	5	MP
F23_PES_2	5.76	PART MIX	LT	20	20	11	MP
F23_PES_3	1.87	PART DI	LT	13	12	6	/
F23_PES_5	1.19	PART DI	LT	11	10	7.5	/
F24_PES_5	11.29	PART DI	SUIT	21		4	U
F25_PES_C	2.19	PART DI	LT	12		11	U
F25_PES_3	2.23	PART DI	LT	15		10.5	U
F26_PES_C	10.52	FULL MIX	SUIT	22	22	9.5	MP
F26_PES_2	8.46	PART MIX	SUIT	21	21	8.5	MP
F26_PES_3	2.39	PART DI	LT	14	14	3	MP
F26_PES_4	6.19	PART DI	LT	18	18	6.5	MP
F29_PES_C	2.72	PART DI	LT	13	8		U
F29_PES_2	17.94	FULL DI	REP	22	22		MP/S
F32_PES_C	2.40	PART SS	LT	11	10	8.5	/
F36_PES_C	8.42	PART MIX	SUIT	21	19		/
F36_PES_1	1.73	PART DI	LT	14	14		MP
F36_PES_4	1.99	PART DI	LT	14	14		MP
F38_PES_C	45.96	FULL SS	REP	22	22	18	MP
F38_PES_1	3.79	PART SS	LT	17	17	13	MP
F38_PES_2	29.55	FULL DI	REP	22	22	19	MP
F38_PES_3	3.52	PART SS	LT	14	14	10	MP
F38_PES_4	13.60	FULL DI	SUIT	22	22	18	MP

F40_PES_C	5.61	PART DI	SUIT	18			U
F42_PES_2	2.63	PART DI	LT	14		6	U
F43_PES_C	1.90	PART DI	LT	10	10		MP
F43_PES_1	1.03	PART DI	LT	15	15		MP
F43_PES_2	1.74	PART DI	LT	16	16		MP
F43_PES_3	3.44	PART SS	SUIT	20	20		MP
F43_PES_4	1.14	PART DI	LT	12	12		MP
F44_PES_C	8.86	PART DI	SUIT	19	19		MP/S
F44_PES_3	34.21	FULL DI	REP	22	22		MP/S
F44_PES_4	7.65	PART SS	SUIT	20	20		MP/S
F44_PES_5	5.00	FULL SS	SUIT	22	22		MP/S
F48_PES_C	1.57	PART DI	LT	16	16		MP
F48_PES_2	2.11	FULL SS	SUIT	22	22		MP
F48_PES_5	5.52	FULL DI	REP	22	22		MP

Of the 48 female volunteers sampled for the male DNA prevalence study, 22 contributed with at least one sample producing useful PP-Y23 profiles. Specifically, nine female volunteers yielded 1 useful Y-STR profile each on 6 fingernail samples collected, four volunteers returned 2 profiles, four volunteers 3, two volunteers 4 and three volunteers yielded 5 useful Y-STR profiles. The volunteers' fingernail length ranged from 0 to 6 mm but did not to play a clear role in the successful detection of male DNA profiles. Among the factors investigated through the questionnaire, the use of shared towels and a handwash frequency lower than ten times a day were associated with the finding of male genetic profiles under volunteers' fingernails. In addition, being involved in an intimate relationship, the use of shared objects, bed-sharing and activities involving holding hands were positively associated with the finding of partners or sons' genetic profiles.

Male DNA transfer and persistence study results

Of the 288 NS collected after scratching, 102 (35.4%) generated Y-STR profiles with 10 or more amplified loci, thus being useful for comparison purposes. The remaining 186 (64.6%) resulted in PP-Y23 profiles with less than 10 Y-STRs detected. Among the 14 NS showing undetermined Y-chromosome quantification results, only 1 returned a useful Y-STR profile, while the remaining 13 resulted in negative PP-Y23 profiles, except for 1 sample with 3 loci and 3 samples with 1 Y-STR detected.

The majority of useful PP-Y23 profiles were found among the t=0 samples that were collected immediately after the scratches. T=0 samples yielded useful Y-STR profiles in 73.6% of the cases (n=53). The profile-quality assignments ranged from reportable full single source or drop-in profiles (n=14; 26.4% of the t=0 samples giving useful profiles) to partial low template profiles (n=22; 41.5%). Of the latter, 8 were characterised as single source, 12 as drop-in and 2 as mixed. Suitable PP-Y23 profiles were observed in 32.1% (n=17) of the useful profiles, being either single source or drop-in full or partial Y-STR profiles (**Tab. 7**).

All 12 female volunteers sampled immediately after the scratching contributed for at least 1 sample a useful PP-Y23 profile. Specifically, six female volunteers yielded 6 useful Y-STR profiles on all 6 fingernail samples collected, one volunteer 5 and one 4, two volunteers yielded 3 useful profiles and the remaining two females only 1. Female volunteers' fingernail length ranged from 0 to 6 mm and showed no apparent effect on the accumulation of male cellular material through scratching while scratching intensity appeared to exert a positive effect in this regard: lighter scratches resulted in less male biological material collected and consequently in Y-STR profiles of lower quality. A picture comparing the results of heavy vs lighter scratching is given in Figure 14.



Figure 14: Pictures of male forearms taken immediately after scratching. Left panel: scratches performed with heavier pressure resulted in a scattered red colouration of the skin; right panel: lighter scratching resulted in a less intense localized red colouration of the skin.

NS PP-Y23 fingernail swab profiles were found to match the scratched male reference Y-chromosome profiles in 88.7% (n=47) of the cases, corresponding to 65.3% of the t=0 group. The associated LR values ranged from hundreds to hundreds of thousands (**Tab. 7**).

The scratched male was excluded as a possible contributor in 6 cases, of which 3 Y-STR low template partial drop-in profiles were incompatible at a single locus only. The remaining 3, mixed or single source low template partial profiles, were incompatible at 1.5 or 2 loci. Females' partners and cohabitants were excluded as possible contributors of the samples not matching the scratched male's Y haplotype. However, some extra alleles detected in mixed and drop-in profiles were found to be compatible with female volunteers' normal contacts (**Tab. 7**).

Autosomal profiling of t=0 NS giving useful PP-Y23 profiles (n=53) resulted in 42 full mixed and 11 drop-in PP-ESX profiles, of which 3 were partial. Among mixed profiles, 14 did not show an amelogenin Y peak while it was detected in 4 drop-in profiles. Among drop-in profiles, 7 turned out to be mixtures after comparison with reference samples, with the female autosomal profile being incomplete for some loci (indicated in Table 7 as (FDO)¹). The total incidence of mixed autosomal profiles accounted for 92.4% (n=49) of the samples analysed.

The scratched male could be included as a possible donor in 69.8% (n=37) of the PP-ESX profiles, including 32 mixed ones (12 as major and 8 as the main contributor) and 5 drop-in profiles. The resulting LR range values ranged from thousands to quadrillions, except for one sample showing an LR of 44 (**Tab. 7**). Autosomal profiling revealed the presence of the scratched male in mixed and drop-in profiles in 2 of the 6 samples that resulted in his exclusion on the basis of Y-STR typing results. The scratched male was excluded as a possible contributor from 16 PP-ESX17 mixed and drop-in profiles. In 11 cases (20.8%) the exclusion derived from the simultaneous absence of the scratched male alleles at some loci and the presence of drop-in alleles possibly deriving from a third contributor. In some instances, the situation was further complicated by allelic dropouts affecting the female volunteer's profile. An example is given in Figure 15.

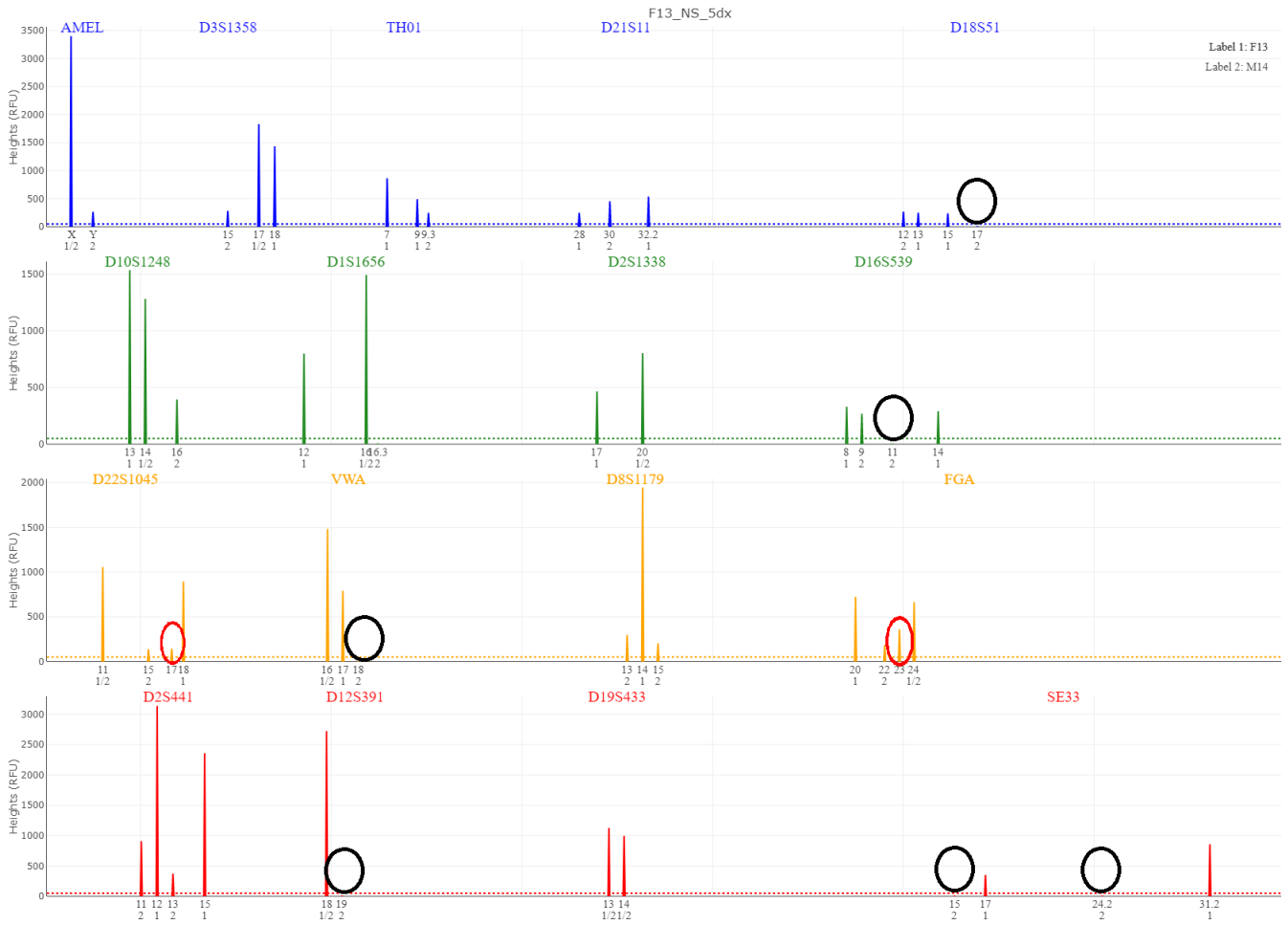


Figure 15: Electropherogram generated through CaseSolver software showing alleles detected with respect to reference samples. Female's alleles are labelled with 1 and scratched male's alleles with 2. In this example, the mixture displays the full profile of the female volunteer, while the scratched male profile shows allelic dropouts in 6 STR loci, highlighted with black circles. In addition, the presence of two extra alleles, highlighted with red circles, possibly deriving from a third minor contributor, leads to the erroneous exclusion of the scratched male as a possible contributor of the sample.

All profiling results obtained for the t=0 NS giving useful Y-STR profiles are summarized in the following Table 7. PP-Y23 and PP-ESX17 profile classification, PP-Y23 profile quality, the number of loci detected and the amount of male DNA, as well as the number of loci compatible with the scratched males, male partners and cohabitants and extra alleles compatibility, are also reported. PP-Y23 and PP-ESX17 matches and LR values obtained from the comparison are included in the table as well.

Table 7: *t=0* NS PP-Y23 and PP-ESX17 typing results for the *t=0* NS producing useful Y-STRs profiles with 10 or more loci amplified. PP-Y23 and PP-ESX17 profiles classification include FULL and PARTIAL single source (SS), drop-in (DI) and mixed (MIX) profiles. For PP-Y23 profiles, profile quality, comprising low template (LT), suitable (SUIT) and reportable (REP) categories, the amount of male DNA (pg/μL) per sample, the number of Y-STR loci detected, the number of compatible Y-STR loci with respect to scratched male (MS), male partner (MP) and male cohabitants (MC) reference Y-STR profiles and information on extra alleles, i.e. those that do not match with the person of interest, are reported. For the duplicated locus DYS385 a score of 0.5 was assigned per each compatible allele. The match with reference profiles, indicated as MS, MP and MC, is shown both for PP-Y23 and PP-ESX profiles as well as LR values. Unknown Y haplotypes and autosomal genetic profiles are reported as “U” in the match columns, and “MP/S” is used when volunteers’ son’s reference Y haplotype was available. Y-STR profile showing one incompatibility with the references are indicated with “/” in the PP-Y23 match column. For PP-ESX17 profiles, autosomal profiles showing female alleles dropouts are designed “(FDO)”¹ in the PP-ESX17 Classification (Class.) column and “+Y” is used to show the presence of a Y signal in drop-in profiles, while “no Y” indicates the absence of a Y signal in mixed profiles. Autosomal incompatibility with the scratched male reference profiles due to situations similar to that reported in Fig. 15 are highlighted with “*” in the PP-ESX 17 match column and the symbol “>” indicate a male autosomal major contribution in the profile.

Sample	PP-Y23 Profiles								PP-ESX17 Profiles				
	Y-quant (pg/μL)	Class.	Prof Qual	Detec Loci	Compatible with:			Extra alleles	Match	LR	Class.	Match	LR
					MS	MP	MC						
F7_NS_4	263.4	FULL SS	REP	22	22				MS	4.40E+04	FULL MIX	F+MS>	5.6E+21
F8_NS_C	22.1	FULL DI	REP	22	22			1U	MS	8.74E+03	FULL MIX	F+MS	5.2E+12
F8_NS_1	3.0	PART DI	LT	13	13			2U	MS	1.88E+04	FULL MIX	F+U*	
F8_NS_2	3.5	PART DI	LT	16	16			1U	MS	4.80E+03	FULL DI (FDO) ¹	MS+U	8.5E+08
F8_NS_3	7.6	PART DI	SUIT	21	21			1U	MS	1.22E+05	FULL MIX	F+MS+U	9.0E+05
F8_NS_4	4.3	PART DI	SUIT	20	20			1U	MS	1.22E+05	PART DI +Y (FDO) ¹	MS+U	3.1E+07
F8_NS_5	3.2	PART DI	SUIT	18	18			1U	MS	2.55E+05	FULL MIX no Y	F+MS+U	2.4E+05
F10_NS_C	84.2	FULL SS	REP	22	22	15	18	1U	MS	2.55E+05	FULL MIX	F+MS+U	4.7E+15
F10_NS_1	12.4	PART SS	SUIT	21	21	14	18		MS	7.50E+04	FULL MIX	F+MS+U	3.8E+09
F10_NS_2	26.0	FULL DI	REP	22	22	15	18	2U, 1MP, 1MC	MS	2.81E+04	FULL MIX	F+MS>+U	2.2E+15
F10_NS_3	55.6	FULL DI	REP	22	22	15	18	1U	MS	2.25E+05	FULL MIX	F+MS+U	2.7E+15
F10_NS_4	90.0	FULL SS	SUIT	22	22	15	18		MS	2.25E+05	FULL MIX	F+MS>	3.8E+23
F10_NS_5	27.2	FULL SS	REP	22	22	15	18		MS	2.25E+05	FULL MIX	F+MS>	1.0E+21
F11_NS_C	9.1	FULL DI	REP	22	22	8.5		1U	MS	3.06E+04	FULL MIX	F+MS>	2.6E+19
F11_NS_1	0.8	PART DI	LT	18	17	8.5		1MP, 1U	/		FULL MIX	F+MS+U	8.1E+10
F11_NS_2	1.1	PART SS	SUIT	19	19	8.5			MS	7.14E+04	FULL MIX no Y	F+MS+U	1.7E+11
F11_NS_3	2.5	FULL DI	SUIT	22	22	8.5		3U	MS	3.06E+04	FULL MIX	F+MS+U	8.5E+20
F11_NS_4	0.9	PART DI	SUIT	19	19	8.5		1U	MS	4.76E+04	FULL MIX	F+MS>+U	2.7E+16

F11_NS_5	3.1	FULL DI	SUIT	22	22	8.5		1U	MS	3.06E+04	FULL MIX	F+MS>	1.5E+17
F13_NS_C	26.8	FULL SS	REP	22	22				MS	4.99E+05	FULL MIX	F+MS+U	1.2E+11
F13_NS_1	3.4	PART SS	LT	12	12				MS	2.75E+03	FULL MIX	F+MS	7.4E+03
F13_NS_2	2.7	PART SS	LT	10	10				MS	1.79E+02	FULL MIX no Y	F+MS	6.7E+05
F13_NS_3	3.8	PART DI	LT	15	14			3U	/		FULL DI +Y (FDO) ¹	MS	3.2E+06
F13_NS_4	3.4	PART DI	LT	17	16			4U	/		PART DI +Y	F	
F13_NS_5	3.1	PART SS	LT	18	18				MS	2.85E+05	FULL MIX	F+U*	
F14_NS_C	3.1	PART SS	SUIT	19	19				MS	2.45E+05	FULL DI +Y	F	
F14_NS_3	3.1	PART DI	LT	17	17			1U	MS	2.75E+03	FULL MIX	F+MS	1.4E+04
F14_NS_4	4.6	PART SS	SUIT	21	21				MS	2.45E+05	FULL MIX	F+MS+U	3.8E+08
F14_NS_5	4.8	PART DI	LT	19	19			3U	MS	2.45E+05	FULL MIX no Y	F+U*	
F24_NS_C	967.6	FULL DI	REP	22	22		10	1MC	MS	4.08E+04	FULL MIX	MS>+U	6.2E+27
F24_NS_1	1.7	PART DI	LT	14	14		7	2U	MS	1.53E+04	FULL MIX	F+U*	
F24_NS_2	1.9	PART SS	LT	10	8.5		4	2U	U		FULL MIX	F+U*	
F24_NS_3	771.2	FULL SS	REP	22	22		10		MS	4.08E+04	FULL MIX	F+MS>+U	1.3E+25
F24_NS_4	3.7	PART SS	LT	19	19		9		MS	2.25E+05	FULL MIX	F+MS+U	6.2E+12
F24_NS_5	25.2	FULL DI	SUIT	22	22		10	1MC, 1U	MS	1.13E+05	FULL MIX	MS+U	2.0E+19
F28_NS_3	2.2	PART SS	LT	10	10	2			MS	4.50E+04	PART DI +Y (FDO) ¹	F+U*	
F28_NS_4	5.8	PART DI	SUIT	21	21	5.5		1U	MS	4.76E+04	FULL DI +Y (FDO) ¹	MS+U	4.4E+22
F28_NS_5	3.4	PART DI	LT	15	15	5		1MP	MS	1.13E+04	FULL DI +Y (FDO) ¹	MS+U	5.535 E+11
F30_NS_C	23.3	FULL DI	SUIT	22	22			2U	MS	5.71E+04	FULL MIX	F+MS+U	8.1E+15
F30_NS_2	7.5	PART DI	LT	17	17			1U	MS	3.57E+04	Full DI (FDO) ¹	F	
F30_NS_3	6.2	PART SS	LT	11	11				MS	1.55E+03	FULL MIX	F+MS	3.9E+12
F30_NS_4	7.1	PART DI	LT	18	18			1 U	MS	2.81E+04	FULL MIX	F+U*	
F30_NS_5	14.3	FULL DI	SUIT	22	22			4 U	MS	2.45E+05	FULL MIX	MS+U	1.4E+19
F36_NS_C	UNDET	PART MIX	LT	17	15	13.5		1 U	U		FULL DI +Y	F	
F40_NS_C	27.9	FULL SS	REP	22	22				MS	2.45E+05	FULL MIX no Y	F+U*	

F40_NS_1	1.3	PART DI	LT	13	13			1 U	MS	7.50E+04	FULL MIX	F+U*	
F40_NS_5	1.7	PART MIX	LT	17	15.5			8 U	U		FULL DI	F	
F41_NS_C	31.0	FULL DI	SUIT	22	22			1 U	MS	7.50E+04	FULL MIX no Y	F+U*	
F41_NS_1	3.7	PART SS	LT	13	13				MS	1.73E+03	FULL MIX	F+U*	
F41_NS_2	6.6	PART DI	SUIT	21	21			1 U	MS	1.18E+04	FULL MIX	MS+U	4.5E+01
F41_NS_3	1024.2	FULL SS	REP	22	22				MS	1.25E+04	FULL MIX	F+MS>	9.3E+24
F41_NS_4	10384.5	FULL SS	REP	22	22				MS	1.25E+04	FULL MIX	F+MS>	1.0E+25
F41_NS_5	149.0	FULL DI	REP	22	22			2 U	MS	2.05E+04	FULL MIX	MS>+U	3.3E+21

Twelve (16.7%) of the 72 NS collected 6 hours after scratching (t=6 group) produced useful Y-STR profiles, all being partial mixed or drop-in. Three (25.0%) of them were classified suitable and the remaining 9 were characterised as low template (**Tab. 8**).

Of the 12 female volunteers sampled 6 hours after scratching, 5 of them contributed with at least one sample producing a useful PP-Y23 profile, three females with 3 samples, one with 2 and one with 1 sample. Fingernail length ranged from 0 to 4 mm and appeared to play no major role in the scratching related accumulation of male biological material.

The Y haplotype of the scratched male was observed in 2 partial, mixed and drop-in, profiles (samples F35_NS_4 and F35_NS_5; Table 8) originating from a single volunteer. They accounted for 16.6% of the t=6 samples giving useful Y-STR profiles and 2.8% of the whole t=6 NS set. The corresponding LR values exceeded 40 thousand. The remaining 10 Y-STR profiles represented unknown Y haplotypes (**Tab. 8**).

None of the activities reported by the female volunteers showed a clear influence on the persistence of male biological material collected by scratching.

Autosomal PP-ESX17 profiling of t=6 samples giving useful Y-STR profiles (n=12) resulted in 5 full mixed profiles (41.7%) of which 3 not showing amelogenin Y signals, 6 drop-in profiles (50.0%) with 3 of them showing an amelogenin Y signal and 1 full single source profile (8.3%) (**Tab. 8**).

The scratched male could not be excluded as a donor in a single case only to the autosomal profile. This particular profile came from one of the two samples showing the scratched male Y haplotype with PP-Y23 typing (sample F35_NS_4). The LR exceeded a value of 400 thousand (**Tab. 8**). The remaining profiles were found to match the female volunteers' autosomal profiles and, in the case of mixtures, unknown individuals.

Table 8 summarises the NS profiling results obtained for the t=6 samples giving useful Y-STR profiles. PP-Y23 and PP-ESX17 profile classification, PP-Y23 profile quality, number of loci detected and the amount of

male DNA, as well as the number of loci compatible with the scratched males, male partners and cohabitants and extra alleles compatibilities are also reported. PP-Y23 and PP-ESX17 matches and LR values obtained are included in the table as well.

Table 8: *t=6 NS PP-Y23 and PP-ESX17 typing results for the t=6 NS producing useful Y-STRs profiles with 10 or more loci amplified. PP-Y23 and PP-ESX17 profiles classification includes FULL and PARTIAL single source (SS), drop-in (DI) and mixed (MIX) profiles. For PP-Y23 profiles, profile quality, comprising low template (LT) and suitable (SUIT) categories, the amount of male DNA (pg/μL) per sample, the number of Y-STR loci detected, the number of compatible Y-STR loci with respect to scratched male (MS), male partner (MP) and male cohabitants (MC) reference Y-STR profiles and information on extra alleles, i.e. those that do not match with the person of interest, are reported. For the duplicated locus DYS385 a score of 0.5 was assigned per each compatible allele. The match with reference profiles, indicated as MS, MP and MC, is shown both for PP-Y23 and PP-ESX profiles as well as LR values. Unknown Y haplotypes or autosomal genetic profiles are reported as “U” in the match columns. In the PP-ESX17 Class. column, “+Y” is used to show the presence of a Y signal in drop-in profiles, while “no Y” indicates the absence of a Y signal in mixed profiles.*

Sample	PP-Y23 Profiles									PP-ESX 17 Profiles			
	Y-quant (pg/μL)	Class.	Prof Qual	Detec Loci	Compatible with:			Extra alleles	Match	LR	Class.	Match	LR
					MS	MP	MC						
F9_NS_C	6.8	PART MIX	LT	15	3.5			20U	U		FULL MIX no Y	F+2U	
F9_NS_3	10.8	PART DI	SUIT	21	6			20U	U		FULL DI	F	
F9_NS_4	2.4	PART SS	LT	13	5			9U	U		FULL DI + Y	F+U	
F17_NS_C	2.3	PART DI	SUIT	19	4			17U	U		FULL DI	F	
F17_NS_4	10.0	PART DI	SUIT	21	8			15U	U		FULL DI + Y	F+U	
F25_NS_C	11.5	PART MIX	LT	19	10.5		12.5	14U	U		FULL MIX no Y	F+U	
F25_NS_3	3.3	PART DI	LT	15	7		5	6U	U		FULL MIX no Y	F+U	
F25_NS_5	1.9	PART DI	LT	12	7		6	4U	U		FULL MIX	F+2U	
F35_NS_3	2.8	PART DI	LT	11	5			9U	U		FULL DI + Y	F	
F35_NS_4	7.0	PART MIX	LT	21	21			5U	MS	2.45E +05	FULL MIX	F+MS	4.6E+ 05
F35_NS_5	1.7	PART DI	LT	11	11			2U	MS	4.50E +04	FULL DI	F	
F37_NS_C	4.0	PART MIX	LT	17	8.5	9.5		10U	U		FULL SS	F	

Among the 72 NS collected 12 hours after the scratches (t=12), 23.6% (n=17) produced useful Y-STR profiles. PP-Y23 profile quality ranged from reportable full mixed profiles, which were obtained for 2 samples (11.8%), to low template partial single source, mixed and drop-in profiles, which accounted for 64.7% (n=11) of the useful profiles. Suitable PP-Y23 profiles were obtained from 4 samples (23.5%), of which 2 were characterised as full mixed and 2 as partial drop-in profiles (**Tab. 9**).

Of the 12 female volunteers sampled 12 hours after scratching, 8 yielded at least one sample producing a useful PP-Y23 profile, including one female with 6 positive samples, one with 4, one with 2 and five females with 1 positive sample each. Fingernail length ranged from 0 to 4 mm and appeared to be unrelated to the accumulation of male biological material underneath fingernails.

Among the 72 t=12 NS Y-STR profiles, the haplotype of the scratched male was found in 2 (11.8%) samples collected from the same female volunteer (samples F38_NS_3 and F38_NS_4; Table 9), which generated two full mixed PP-Y23 profiles. These 2 samples corresponded to 2.8% of the whole t=12 NS set. The LR values ranged from 1700 to 460 thousand (**Tab. 9**). One of the two mixtures (sample F38_NS_3) was fully compatible also with the Y haplotype of the female volunteer's partner, while from the other (sample F38_NS_4) the partner was excluded on bases of 1.5 incompatible allele calls. Haplotypes of partners of female volunteers were also found in another 6 t=12 samples, of which one was a mixture with the Y profile of a male cohabitant. The altogether 7 mixed Y-STR profiles matching the Y haplotype of female volunteers' partners accounted for 9.7% of the entire NS t=12 sample pool.

Activities involving spending time with the male partner and engaging in cuddling and/or bed-sharing appeared to have had a mildly positive effect on the detection of the partners' genetic profiles. In addition, none of the self-reported female volunteers' activities showed a clear influence on the persistence of the scratched males' biological material underneath the fingernails.

Autosomal PP-ESX17 profiling of the 17 t=12 samples with useful Y-STR profiles resulted in 6 full single source autosomal profiles (35.3%) matching the female volunteers' profiles, 6 full mixed profiles (35.3%), with 3 of them without an amelogenin Y signal, and 5 full drop-in profiles (29.4%) (**Tab. 9**).

Among these 6 PP-ESX17 mixed profiles, the profile of the scratched male was observed in 2 samples collected from the same volunteer (samples: F38_NS_4 and F38_NS_5), of which one revealed the scratched male's autosomal profile mixed with the female's profile. This sample (F38_NS_4) also yielded the mixed Y-STR profile matching the scratched male Y haplotype. Whereas the other sample (F38_NS_5) revealed the scratched male's partial autosomal profile in conjunction with the female volunteer's and her male partner's profiles. However, by Y-STR typing the scratched male was excluded as a possible contributor for being not compatible at one locus. LR values for the two autosomal profiles matching the scratched male ranged from 50 thousand to billions (**Tab. 9**). Volunteers' partner autosomal profiles were observed in another 2 mixed PP-ESX17 profiles, including one not showing an amelogenin Y signal. The altogether 3 samples matching the female volunteers' partners accounted for 4.2% of the NS t=12 set.

Table 9 summarises the NS profiling results obtained for the t=12 samples giving useful Y-STR profiles. PP-Y23 and PP-ESX17 profile classification, PP-Y23 profile quality, number of loci detected and the amount of male DNA, as well as the number of loci compatible with the scratched males, male partners and cohabitants and extra alleles compatibilities are also reported. PP-Y23 and PP-ESX17 matches and LR values obtained from the comparison are included in the table as well.

Table 9: *t=12 NS PP-Y23 and PP-ESX17 typing results for the t=12 NS producing useful Y-STRs profiles with 10 or more loci amplified. PP-Y23 and PP-ESX17 profiles classification includes FULL and PARTIAL single source (SS), drop-in (DI) and mixed (MIX) profiles. For PP-Y23 profiles, profile quality, comprising low template (LT), suitable (SUIT) and reportable (REP) categories, the amount of male DNA (pg/μL) per sample, the number of Y-STR loci detected, the number of compatible Y-STR loci with respect to scratched male (MS), male partner (MP) and male cohabitants (MC) reference Y-STR profiles and information on extra alleles, i.e. those that do not match with the person of interest, are reported. For the duplicated locus DYS385 a score of 0.5 was assigned per each compatible allele. The match with reference profiles, indicated as MS, MP and MC, is shown both for PP-Y23 and PP-ESX profiles as well as LR values. “np” in the PP-Y23 LR column indicates that the LR calculation was not possible through the YHRD. Unknown Y haplotypes or autosomal genetic profiles are reported as “U” in the match columns, and “MP/S” is used when volunteers’ son’s reference Y haplotype was available. Y-STR profile showing one incompatibility with the references are indicated with “/” in the PP-Y23 match column. In the PP-ESX17Class. column “+Y” is used to show the presence of a Y signal in drop-in profiles, while “no Y” indicates the absence of a Y signal in mixed profiles. Autosomal incompatibility with the scratched male reference profiles due to situations similar to that reported in Fig. 15 are highlighted with “*” in the PP-ESX 17 match column.*

Sample	PP-Y23 Profiles									PP-ESX17 Profiles			
	Y-quant (pg/μL)	Class.	Prof Qual	Detec Loci	Compatible with:			Extra alleles	Match	LR	Class.	Match	LR
					MS	MP	MC						
F1_NS_C	4.3	PART DI	SUIT	19	6.5	19		1U	MP/S	5.63E+04	FULL SS	F	
F2_NS_C	4.5	PART MIX	LT	18	12	12		9U	U		FULL DI	F	
F2_NS_2	1.4	PART DI	LT	12	5	7		2U	U		FULL SS	F	
F2_NS_3	5.0	PART MIX	LT	18	12.5	9		13U	U		FULL DI	F	
F2_NS_4	5.6	PART MIX	LT	18	9	7		13U	U		FULL SS	F	
F5_NS_C	0.1	PART DI	LT	11	2			9U	U		FULL SS	F	
F19_NS_4	1.3	PART SS	LT	10	5			5U	U		FULL MIX no Y	F+U	
F32_NS_2	1.7	PART SS	LT	10	3	10	7		MP	9.00E+03	FULL SS	F	
F33_NS_C	3.1	PART SS	LT	17	4	17			MP	3.57E+04	FULL MIX	F+MP	6.0E+01
F33_NS_2	1.1	PART SS	LT	13	3	13			MP	2.25E+05	FULL MIX no Y	F+MP+U	2.6E+02
F38_NS_C	32.8	FULL MIX	SUIT	22	22	22	20.5	1MC, 2U	MP+MC	1394	FULL DI + Y	F	
F38_NS_1	2.5	PART DI	LT	13	8	12	8	2U	/		FULL SS	F	
F38_NS_2	6.4	FULL MIX	SUIT	22	17	22	20.5		MP	np	FULL DI + Y	F	
F38_NS_3	51.2	FULL MIX	REP	22	22	22	19.5		MS+MP	1791	FULL MIX no Y	F+U *	
F38_NS_4	50.9	FULL MIX	REP	22	22	20.5	18.5		MS	6.20E+03	FULL MIX	F+SM	5.8E+04
F38_NS_5	6.9	PART DI	SUIT	19	18	13	11		/		FULL MIX	F+MP+SM	2.2E+12
F42_NS_C	4.2	PART MIX	LT	17	8		16	2U	/		FULL DI	F	

Of 72 NS collected 24 hours after scratching ($t=24$), 20 (27.8%) produced useful PP-Y23 profiles. Y-STR profile quality ranged from suitable -full or partial- single source, drop-in and mixed profiles obtained from 60% ($n=12$) of the $t=24$ samples giving useful Y-STR profiles, to low template partial single source, drop-in and mixed profiles obtained in 35% of the cases ($n=7$). Notably, only 1 reportable PP-Y23 profile, being characterised as full mixed, was obtained from the 20 $t=24$ samples giving useful Y-STR profiles (**Tab. 10**).

Of the 12 female volunteers sampled 24 hours after scratching, 6 of them contributed with at least one sample that generated a useful PP-Y23 profile, including two females with 5 positive samples, two with 4 and 2 with one positive sample each. Fingernail length ranged from 0 to 5 mm and appeared to be unrelated to the accumulation of male biological material underneath fingernails.

Among the 20 $t=24$ samples yielding useful Y-STR profiles, the Y haplotype of the scratched male was found in 2 (10%) samples collected from the same female volunteer (samples: F44_NS_C and F44_NS_4), corresponding to 2.8% of the whole $t=24$ NS set. These resulted in two full mixed PP-Y23 profiles, of which one provided an LR higher than 10 thousand, while for the other the LR calculation through the YHRD was not possible (indicated as “np” in Table 10). One of these Y haplotype mixtures (F44_NS_4) was found to be fully compatible also with the volunteer partner’s or son’s Y haplotype, while from the other, the partner/son was excluded for 3 incompatible loci (F44_NS_C). Female volunteer partners’ haplotypes were also found in another 11 $t=24$ samples, of which 2 could have derived from the genetic contribution of the volunteer’s son. The altogether 12 samples matching the volunteers’ partner/son’s haplotype accounted for 16.7% of the entire $t=24$ NS set, including one mixed Y-STR profiles showing also the Y-chromosome profile of the volunteer’s male cohabitant (F26_NS_3).

The 2 samples showing a contribution of the scratched male in the $t=24$ group derived from a single volunteer that reportedly did not wash her hands within the last 10 hours before the sampling. Finding the genetic profiles of the male partner/offspring might have been related to activities that occurred between the scratching and the sampling of the fingernails. The list of such activities includes the use of objects shared with a male partner and/or children, baby care, time spent with the male partner by engaging in cuddling and/or sharing the bed, as well as sexual intercourse.

Autosomal PP-ESX17 profiling of $t=24$ samples giving useful Y-STR profiles ($n=20$) resulted in 2 full single source (10%) and 11 drop-in autosomal profiles (55.0%; 2 of which showing an amelogenin Y peak), matching the female volunteers’ profiles. Full mixed profiles were obtained for 7 samples (35%) with 2 of them being without an amelogenin Y signal (**Tab. 10**).

The profile of the scratched male was not observed among PP-ESX17 mixed profiles. This holds true also for sample F44_NS_C, for which the male’s contribution had to be considered non-informative due to the simultaneous absence of the scratched male’s alleles at some loci and the presence of drop-in alleles (see Table 10), a situation similar to that already reported for some $t=0$ samples (see Fig. 15). In addition, also the volunteer’s partner was excluded from 1 PP-ESX17 mixed profile (sample F26_NS_1) for the same reasons

while showing a matching haplotype by Y-STR typing. The volunteers' partner's autosomal profile was found in 2 mixed autosomal profiles (samples: F22_NS_C and F26_NS_3; Table 10), of which 1 being compatible also with the volunteer's male cohabitant's profile (F26_NS_3). The latter sample produced a Y haplotype mixture of the partner's and cohabitant's PP-Y23 profiles. Moreover, 1 mixture (F43_NS_C) was found to match the volunteer's daughter's profile, while the remaining 2 (samples: F44_NS_5 and F48_NS_3; Table 10) showed the female autosomal profiles mixed with unknown individuals.

Table 10 summarises NS profiling result obtained for the t=24 giving useful Y-STR profiles. PP-Y23 and PP-ESX17 profile classification, PP-Y23 profile quality, number of loci detected and the amount of male DNA, as well as the number of loci compatible with the scratched males, male partners and cohabitants and extra alleles compatibilities are also reported. PP-Y23 and PP-ESX17 matches and LR values obtained from the comparison are included in the table.

Table 10: t=24 NS PP-Y23 and PP-ESX17 typing results for the t=24 NS producing useful Y-STRs profiles with 10 or more loci amplified. PP-Y23 and PP-ESX17 profiles classification includes FULL and PARTIAL single source (SS), drop-in (DI) and mixed (MIX) profiles. For PP-Y23 profiles, profile quality, comprising low template (LT), suitable (SUIT) and reportable (REP) categories, the amount of male DNA (pg/μL) per sample, the number of Y-STR loci detected, the number of compatible Y-STR loci with respect to scratched male (MS), male partner (MP) and male cohabitants (MC) reference Y-STR profiles and information on extra alleles, i.e. those that do not match with the person of interest, are reported. For the duplicated locus DYS385 a score of 0.5 was assigned per each compatible allele. The match with reference profiles, indicated as MS, MP and MC, is shown both for PP-Y23 and PP-ESX profiles as well as LR values. "np" in the PP-Y23 LR column indicates that the LR calculation was not possible through the YHRD. Unknown Y haplotypes or autosomal genetic profiles are reported as "U" in the match columns, "MP/S" is used when volunteers' son's reference Y haplotype was available, and "D" is used for daughter. Y-STR profile showing one incompatibility with the references are indicated with "/" in the PP-Y23 match column. In the PP-ESX17 Class. column "+Y" is used to show the presence of a Y signal in drop-in profiles, while "no Y" indicates the absence of a Y signal in mixed profiles. Autosomal incompatibility with the scratched male or male partner reference profiles due to situations similar to that reported in Fig. 15 are highlighted with "*" in the PP-ESX 17 match column.

Sample	PP-Y23 Profiles									PP-ESX 17 Profiles			
	Y-quant (pg/uL)	Class.	Prof Qual	Detec Loci	Compatible with:			Extra alleles	Match	LR	Class.	Match	LR
					MS	MP	MC						
F22_NS_C	8.2	PART DI	SUIT	18	6	18		3U	MP	2.45E+05	FULL MIX	F+MP+U	3.0E+02
F22_NS_1	2.1	PART SS	LT	13	2	12		1U	/		FULL DI	F	
F22_NS_3	2.4	PART DI	LT	15	6.5	15		1U	MP	2.45E+05	FULL SS	F	
F22_NS_4	3.6	PART SS	LT	12	4.5	12			MP	2.69E+03	FULL DI	F	
F26_NS_C	8.8	PART DI	SUIT	20	7	20	7.5	2MC,1U	MP	1.44E+04	FULL DI	F	
F26_NS_1	10.0	FULL SS	SUIT	22	8	22			MP	9.51E+04	FULL MIX no Y	F+U*	
F26_NS_2	15.1	FULL DI	SUIT	22	12	22		4MS	MP	1.22E+05	FULL DI	F	

F26_NS_3	10.5	FULL MIX	SUIT	22	10	22	12.5	5MC	MP+MC	np	FULL MIX	MP+MC	1.27E+06
F26_NS_4	7.8	PART SS	SUIT	19	7	22			MP	2.38E+04	FULL SS	F	
F29_NS_C	1.2	PART DI	LT	10	6	8		4U	U		FULL DI + Y	F	
F43_NS_C	2.9	PART DI	SUIT	21	6.5	21		2U	MP	4.50E+04	FULL MIX no Y	F+D	1.2E+21
F44_NS_C	18.2	FULL MIX	REP	22	22	19			MS+U	np	FULL MIX	F+U*	
F44_NS_2	1.6	PART SS	LT	10	2	10			MP/S	6.27E+04	FULL DI	F	
F44_NS_3	17.7	PART MIX	SUIT	21	18	21		7U	MP/S+U	374	FULL DI	F	
F44_NS_4	35.5	FULL MIX	SUIT	22	22	22		3U	MS+MP/S	1.00E+04	FULL DI + Y	F	
F44_NS_5	2.9	PART MIX	LT	15	11	13.5			U		FULL MIX	F+U	
F48_NS_C	2.3	PART MIX	SUIT	21	7	20		13U	/		FULL DI	F	
F48_NS_1	1.0	PART DI	LT	17	4	10		7U	U		FULL DI	F	
F48_NS_2	6.7	PART MIX	SUIT	20	3	16		10U	U		FULL DI	F	
F48_NS_3	2.1	PART DI	SUIT	21	8	19.5		3U	U		FULL MIX	F+U	

Discussion

In violence against women investigations, the collection of fingernail debris may provide valuable biological evidence amenable to DNA profiling, and ultimately lead to the identification of the perpetrator and elucidation of the criminal circumstances. Indeed, in both sexual and non-sexual assaults against women, usually committed by men, the close and violent physical contact between the victim and perpetrator may lead to the accumulation of genetic material under the protected fingernail's hyponychial area, thus providing biological evidence that may be analysed by DNA typing for court proceedings. In this context, the main objective of the present thesis was to study the mechanisms of male DNA transfer, persistence, prevalence and recovery under fingernails of women under a mock physical assault scenario.

Forty-eight female volunteers were enrolled for a controlled scratch study involving superficial scratches performed on males' forearms mimicking a defensive action during a physical assault. Scratches were performed after the collection of fingernail control samples (CS) after vigorous cleaning of the hyponychial area to reduce possible persisting genetic backgrounds and assess the level of transfer of male DNA under fingernails resulting from scratches. Targeted sampling of the fingernail debris was performed at regular time intervals after the scratching to explore the persistence of male cellular material following the mock physical assault. Furthermore, information regarding factors potentially affecting the post-assault detection of male DNA was collected by means of a questionnaire.

Fingernail samples were collected from female volunteers two weeks ahead of the actual scratch experiment to investigate the background level, or prevalence, of male DNA under the volunteers' fingernails in normal daily life. This was done to compare non-crime related everyday DNA transfers beneath fingernails with transfers under mock assault circumstances, for identifying factors that may have led to or influenced the detection of foreign DNA profiles of biological material acquired in the two aforementioned possible scenarios.

Two different sampling strategies for the collection of fingernail samples were examined, since different methods may influence the amount of foreign DNA being recovered from the fingernails' hyponychial areas. From all the female volunteers, fingernail debris from one hand was collected cumulatively by using a single sterile cotton swab for all five fingernails, whereas the collection of the other hand occurred in a finger by finger fashion, using one sterile cotton swab per finger. To reduce possible confounding factors, for half of the volunteers the cumulative collection was performed on the dominant hand and finger by finger from the non-dominant one and *vice versa* on the other half of the volunteers.

As a rule of thumb, male DNA quantification data on the entire dataset showed statistically significantly higher male DNA yields for the cumulative collection method, a finding already pointed out in the literature [93]. By contrast, statistically significant differences in male DNA yield were found neither among the individual fingers nor between the dominant and non-dominant hand. However, in the present study, the

cumulative collection not always resulted in significantly higher male DNA yields and good quality profiles, including reportable and suitable Y-STR profiles, were also obtained with swabs taken from individual fingers. Significant differences in male DNA content observed in the PES sampling group between cumulative and finger by finger collection of the non-dominant hand (**Fig. 13**) may reflect a possibly lower accumulation of exogenous DNA under the fingernails of this hand. This might result from a reduced involvement of the non-dominant hand in daily activities that lead to the collection of foreign biological material. Furthermore, differences in the scratching related collection of foreign genetic material may be expected among the different fingers of a hand. For the NS set, the difference in male DNA content between samples collected cumulatively and from finger 1 (i.e. the thumb) of the non-dominant hand may have arisen from the execution of the scratching -the thumb may have been less involved, and hence it may have picked up lower amounts of male DNA. Interestingly, this finding was restricted to the thumb of the non-dominant hand, which might indicate that for finger 1 scratching with the dominant hand could be more effective in collecting male cellular material.

For real-life forensic casework, these findings suggest that under normal non-crime related circumstances a cumulative collection may be worth for the recovery of exogenous genetic material beneath fingernails of the non-dominant hand. However, for criminal cases, a finger by finger collection appears to be a better choice as it leads to comparable yields of exogenous male DNA while avoiding the potential creation of mixed stains comprising biological material that was originally found under single fingernails only. In addition, in the course of this study, cumulative collection sometimes led to a partial loss of available biological debris because of “swab saturation” by high debris quantities. In these occasions, a finger by finger collection should be performed and a sterile tube may be used for collecting voluminous parts of debris while swabbing the area.

For the prevalence study, 37.5% of the PES collected two weeks before the actual scratch experiment contained sufficient amounts of male DNA to proceed with Y-STR typing. The establishment of a male DNA quantification cut-off was found to be convenient for avoiding the futile typing of samples falling short of male DNA amounts being required for successful Y-STR profiling. Application of such a cut-off may also be attractive in real forensic casework, as it allows focusing on the analysis of relevant samples while saving valuable DNA extracts.

The prevalence of male cellular material beneath volunteers’ fingernails acquired under non-(mock)assault circumstances revealed by the present study (37.5%) was found to be higher than the prevalence of foreign DNA observed under fingernails of the general population [77] and to be similar to that reported for cohabiting couples [81]. However, previous studies investigated the prevalence of exogenous DNA through autosomal profiling rather than focusing on male DNA. This is potentially problematic. In a study also performing Y-STR profiling [81], 63% of the samples yielding single source autosomal STR profiles turned out to contain also a male contribution. Among the 108 PES qualifying for Y-STR analysis, 17.7% (n=51) gave useful Y-STR profiles with 10 or more Y-STRs being amplified. Six of them (11.8%) were of

remarkable quality and defined reportable, 27.4% (n=14) had good quality and were defined as suitable. However, the majority of samples (n=31, 60.8%) produced poor quality Y-STR profiles that were defined as low template. These rates are again higher than those reported in the literature for finding of good quality exogenous autosomal STR profiles, being defined as high level or reportable mixture [77-79, 81]. This finding underlines the versatility of Y-STR typing for samples containing male/female mixtures and demonstrates the method's reliability in selectively amplifying and typing minute amounts of male DNA, even in the presence of high amounts of female DNA [50, 72].

Among the 48 female volunteers participating in this study, 24 reported being involved in an intimate relationship with a man. Twelve of them were cohabiting with their male partner, including 3 of them also cohabiting with their sons. In addition, 10 volunteers reported to cohabit with other family members including brothers and fathers, 15 to live alone and the remaining 11 volunteers reported to share their accommodation with friends or colleagues with only one of them living with a male.

Of the 51 PES PP-Y23 profiles, 56.9% (n=29) were found to match the volunteers' partners or sons' Y haplotypes. Specifically, partners' Y-STR profiles were found among 15 reportable and suitable PP-Y23 profiles (5.2%), of which 5 could have also derived from a genetic contribution of the son, and in 14 low template Y-STR profiles. Unknown Y haplotypes were observed in 43.1% (n=22) of the Y-STR profiles, of which 5 were defined reportable and suitable (1.7%). Interestingly, among these PP-Y23 profiles showing unknown Y haplotypes, 5 -classified as low template- were found to not match with the partner's Y-STR haplotype on basis of a single incompatibility. In these cases, low amounts of male DNA resulted in poor quality Y-STR profiles, being affected by stochastic effects such as dropouts of the contributor alleles and drop-in of extra alleles. This led to the exclusion of the person of interest. Another PP-Y23 profile, being a mixture and classified suitable, showed two incompatibilities with the partner's Y-STR reference haplotype. In this specific case, the presence of more than one contributor with the simultaneous absence of the partner's alleles at two loci led to his exclusion as a possible contributor.

Twenty-two of the 48 female volunteers sampled for the male DNA prevalence study, contributed at least for one sample a useful PP-Y23 profile. Among factors investigated through the questionnaire, the use of shared towels and a handwash frequency lower than ten times a day were associated to the finding of male genetic profiles under the volunteers' fingernails, while being involved in an intimate relationship, cohabiting with males, the use of shared objects, bed-sharing and activities involving holding hands, were positively associated to finding the partner's or son's Y haplotype.

The results obtained from the prevalence study showed that women involved in an intimate heterosexual relationship had -regardless of the cohabiting status- a greater chance to acquire partner's genetic profiles under their fingernails, and the cohabitation with a man increased the chance for observing male-specific genetic profiles as well as Y-haplotype mixtures. Therefore, in violence against women cases, the interpretation of genetic profiles found under the victim's fingernails should be carefully performed and the

possibility of a non-assault related transfer of genetic material has to be taken into account. This is particularly true for profiles matching with those of the victim's intimate partner or cohabitants.

The transfer and persistence study revealed an incidence of 35.4% (n=102) of useful Y-STR profiles obtained from fingernail samples collected after scratching (NS). With respect to the prevalence of useful PP-Y23 profiles obtained from PES in the prevalence study (17.7%), this result, being in line with previous scratch studies [79, 82, 83], demonstrates that mock defensive scratching constitutes a realistic model for the transfer of male biological material underneath the victim's fingernails. This statement is further backed by the fact that the observed incidence of useful Y-STR profiles turned out to be consistent with data from real casework [79, 90], although no statistically significant differences were found among male DNA quantification values when comparing the PES and NS sample sets. However, both the PES and the NS set showed statistically significantly higher male DNA contents than the CS set, which was collected from the cleaned hyponychial area directly before the actual scratching took place. This demonstrates that the cleaning procedure was effective in reducing the baseline male genetic background being present under the fingernails of the female volunteers. Thus genetic results obtained for NS reflect the DNA transfer derived from the mock defensive scratching (t=0 samples) and/or from non-crime related transfers that occurred between the scratching and the delayed sampling (t=6, 12 or 24 h post scratching).

The majority of useful PP-Y23 profiles were found among t=0 samples collected immediately after scratches compared to the samples collected with 6-, 12- and 24-hours delay, although a statistically significant difference was not found. With t=0 sampling, 73.6% (n=53) of the collected samples yielded useful Y-STR profiles with 26.4 % (n=14) giving reportable Y-STR profiles, 32.1% (n=17) suitable and 41.5 % (n=22) partial low template profiles (**Tab. 7**).

Notably, all of the 12 female volunteers sampled immediately after the scratching, produced at least for one sample a useful PP-Y23 profile. The intensity applied by volunteers during scratching was the determining factor for successful Y-STR typing: lighter scratching resulted in lower male DNA yields and in poor quality or not useful Y-STR profiles (**Fig. 14**). This result is in agreement with findings of a previous scratch study [79].

The scratched male's reference Y-chromosome haplotype was found in 88.7% (n=47) of the useful t=0 Y-STR profiles, corresponding to 65.3% of the entire t=0 NS set. The very high LR values, ranging from hundreds to hundreds of thousands, would have strongly supported the prosecutor's hypothesis (**Tab. 7**). However, in this context, it should be mentioned that LR values rely on the validity of the Y haplotype frequencies in the reference population sample, which were obtained through the YHRD database.

The exclusion of the scratched male as a possible contributor of 6 Y-STR profiles resulted from poor profile quality. Indeed, these 6 low template partial, drop-in and mixed profiles were found to feature one to two incompatibilities with the scratched male's reference Y haplotype. These incompatibilities were caused by

stochastic effects that led on the one hand to allelic dropout and on the other hand to drop-in of extra alleles. In 5 t=0 NS Y-STR profiles, extra alleles were found to be compatible with those of the volunteers' male partners or cohabitants (**Tab. 7**), suggesting a possible persistence of alleles deriving from volunteers' daily life contacts. In addition, unknown extra alleles were also observed, and these may have been acquired during normal daily life activities, e.g. working with young children or visiting patients. However, it is also possible that extra alleles may have derived from trace DNA being present on the skin of the scratched male.

Autosomal profiling of t=0 NS giving useful PP-Y23 profiles resulted in 42 full mixed and 11 drop-in PP-ESX17 profiles, of which 7 turned out to be mixtures being the female autosomal STR profile detected at a few loci only (indicated by using "(FDO)¹" in Table 7).

The autosomal profile of the scratched male was observed in 32 mixed and 5 drop-in PP-ESX17 profiles. The scratched male was identified 12-times as major and 8-times as the main contributor to the mixed profile. Hence, the scratched male was considered a possible donor in 69.8% of the analysed samples, with LR values ranging from thousands to quadrillions. The only exception was a single sample with an LR of 44, which still would have been in support of the prosecutor's hypothesis. Notably, the scratched male was included as the main contributor to 5 of the 7 (FDO)¹ drop-in profiles, since the volunteer's alleles were observed as drop-ins. In these cases, the LR calculation was not conditioned on the volunteer profile (indicated in Table 7 as MS+U). An LR calculation not conditioned on the female volunteer was also performed for another 5 mixed autosomal profiles, of which 2 showed the scratched male as the major contributor with some volunteer's alleles dropping out and extra alleles observed as well. Finally, the scratched male was excluded as a possible contributor to 16 PP-ESX17 mixed and drop-in profiles. However, in 11 cases (20.8%) the exclusion was due to the simultaneous absence of the scratched male's alleles at some loci and the presence of drop-in alleles possibly deriving from a third contributor (**Fig. 15**). In some instances, this situation was further complicated by allelic dropouts affecting the female's profile, which led to the generation of autosomal profiles with no more than 4 alleles per locus. Thus, the LR calculation would have not supported the hypothesis of the scratched male being a contributor if conditioned on the female volunteer's profile, although his profile was present and detected.

The percentage of useful Y-STR profiles (73.6%) obtained from samples collected immediately after scratching (t=0) was lower than previously reported values [83]. This may be explained with the sampling approach utilized in the present study, which involved not only cumulative but also finger by finger collection. The latter resulted in differing male DNA yields for the individual fingers and consequently in a higher rate of not useful Y-STR profiles. Notably, the percentage of mixed autosomal profiles obtained for the 53 analysed t=0 samples (92.4%) was higher than previously reported values [79]. This might be explained by a different scratch intensity strategy applied in the previous study but also by the fact that in the present study fingernail samples were first analysed for Y-STRs and only samples producing useful PP-Y23 profiles were then processed for autosomal profiling, thus indicating the advantage of a preliminary Y-STR

testing which may be beneficial in real forensic casework to focus on relevant samples while saving valuable DNA extracts.

The persistence of scratch related male cellular material under the volunteers' fingernails greatly decreased within the first 6 hours after the scratching, being lost during normal daily activities. This finding is in agreement with previous research [79, 83]. Nevertheless, PP-Y23 profiles matching with the scratched male's reference Y-STR haplotype were obtained from the fingernail debris of three female volunteers that were sampled respectively 6, 12 or 24 hours after the scratching. In each case, 2 samples (accounting for 2.8% of the respective sample pool) produced the mock perpetrator's genetic fingerprint. For sexual assault cases, these results clearly speak in favour of a collection of fingernail debris in case of victim death as the lack of activities may preserve the cellular material under the fingernails [87], but also in cases of victim survival, the collection of fingernail samples can be valuable as well, even if the time lag between the crime and the sampling is high. Twelve and 24 hours after scratching, a rise in the detection rate of Y-STR haplotypes deriving from the volunteers' male partners was observed, reaching frequencies similar to that observed in the prevalence study for the $t=12$ samples (9.7%). For the $t=24$ samples this proportion was even higher (16.7%). A similar picture was obtained for the volunteers' sons and male cohabitants. Obviously, this phenomenon has a high impact on the interpretation of the genetic findings and strongly suggests that such data need to be interpreted with great caution and, if possible, considering reference profiles of female's normal contacts.

In relation to the finding of the scratched male's Y haplotype among $t=6$ and $t=12$ samples, none of the activities reported in the questionnaire could explain the higher persistence of the male genetic material. However, for the $t=24$ samples refraining from handwashing within the last 10 hours before the sample collection was positively associated with the recovery of the mock perpetrator's genetic profile under the mock victim's fingernails.

Overall, autosomal STR profiling results obtained from NS $t=0$ subset demonstrated the intricacies of forensic trace DNA analysis of fingernail debris. Despite the fact that $t=0$ sampling took place immediately after the scratching, the resulting genetic fingerprints were typically complex and non-trivial to interpret. Nevertheless, autosomal STR profiling allowed for the detection of the scratched male's profile in a mixed and a drop-in profile for 2 out of 6 samples with Y-STR results suggesting the mock perpetrator's exclusion. Generally, the LR values distinctly exceeded those obtained with Y-STR profiling alone, which underlines the importance of targeting both typing STR marker systems in real forensic casework on sexual assault cases as findings may complement and enhance each other [72]. The interpretation of autosomal STR profiles obtained 6, 12 and 24 hours post scratching exhibited an even higher degree of complexity, however, the scratched male genetic profile was observed respectively in 1, 2 and 0 $t=6$, $t=12$ and $t=24$ samples. The complexity of such profiles was basically due to the increase in the detection of genetic profiles deriving from volunteers' normal contacts, determining the simultaneous presence of drop-in alleles deriving from the

third contributor as well as the absence of the scratched male's alleles at some loci, thus the scratched male's contribution had to be considered non-informative.

The use of the CaseSolver software [104] was found to be not as effective as hoped for. Automatic detection of matches with the scratched males' reference profiles was achieved in a mere 22 cases where the mock perpetrator constituted the major or main contribution to the mixed stain or when his alleles were detected at all loci. In the remaining cases, the genetic fingerprint of the scratched male was assigned to match an unknown source. Nonetheless, the software was a great enhancement in the final interpretation of fingernail profiles and in the LR calculations. It provides a user-friendly interface, enables storage of the genetic profiles of traces and reference samples, and offers different options for the visualization of genetic profiles and their comparison with reference samples. Finally, it also provides the opportunity to compute LR values and testing different alternative hypotheses directly from the CaseSolver interface, by using the EuroForMix software [105].

In a general perspective, results from the persistence study underlined the importance of collecting fingernail evidence as soon as possible after the assault, particularly for survivors. The foreign genetic material deriving from criminal actions may and will be lost during normal daily life activities and, most importantly, non-assault related foreign genetic material may be acquired over time as well. Particularly, settings involving post-assault activities such as intercourse with a consensual intimate partner carry a high risk of creating mixed stains or increasing their complexity, which complicates profile interpretation. This result is in agreement with research on other sexual assault samples, where discrepancies between the victim's account and genetic findings have been reported. This particularly holds true for cases with distinctly delayed sampling, which led to the detection of DNA profiles of individuals not being related to the crime but involved in an intimate relationship with the victim [107, 108]. If the assault results in femicide, the lack of activities may preserve the male cellular material under the fingernails [85-87]. Hence, the sampling of fingernails should always be considered under such circumstances.

A clear demonstration on the ease of acquiring foreign DNA under fingernails in normal daily life circumstances has been revealed from the 2 PES samples that derived from a contamination event. In 2 samples collected from two different volunteers, the same Y-STR haplotype was observed, which subsequently was found to belong to the child of a colleague. A couple of days ahead of the sampling of the two affected volunteers, the boy was waiting for his mother in the lab corridor where the fingernail samplings took place. He was playing with the chair on which volunteers sat during the fingernail sample collection. He probably left some biological material, possibly under the chair, which the two volunteers unintentionally picked up. This ultimately led to the detection of his Y-STR haplotype under their fingernails. This contamination occurred despite the use of DPI, decontamination of surfaces and the use of sterile disposable devices, which demonstrates the exquisite sensitivity of the applied genotyping approaches and highlights the need for rigorous standard operating procedures for contamination avoidance and management. This is of particular importance when not specifically trained practitioners or nurses perform

the collection of fingernail samples in hospitals. However, to avoid sample contamination, leading in the worst case to miscarriages of justice, this also applies to forensic genetics laboratories. In addition to the adoption of standard operating procedures for fingernail evidence collection and preservation and the use of sterile single-use devices in defined and constantly monitored work areas, the training of lab personnel and the establishment of a staff elimination database are crucial elements of best laboratory practice.

Conclusion

The present study confirms that the interpretation of genetic findings from fingernail samples, as any other trace DNA evidence, can be challenging, but valuable insight can be expected at the same time.

Male DNA may be acquired under fingernails during normal, everyday contacts. Furthermore, women involved in an intimate relationship -regardless of the cohabiting status- have a greater chance to acquire partner's genetic profiles under their fingernails. Alike, cohabitation with a male increases the chance to detect male-specific genetic profiles and Y-haplotype mixtures, which complicates the interpretation of forensic genetic findings.

The chosen mock crime scenario resulted in a realistic model for the transfer of male DNA under the volunteers' fingernails, leading to the detection of the DNA profile of the scratched male in 88.7% of the useful t=0 Y-STR haplotypes. However, fingernail PP-Y23 profile interpretation was complicated by poor profile quality, which was characterised by stochastic effects and the presence of extra alleles, possibly deriving from the female volunteers' previous normal contacts and activities. In some instances this prevented the unequivocal identification of the scratched male's Y-STR haplotype.

Moreover, the interpretation of autosomal STR profiles exhibited an even higher degree of complexity. In some cases the scratched male was found to be the major or main contributor to the genetic profiles. However, in other cases the simultaneous absence of the scratched male's alleles at some loci and the presence of drop-in alleles, possibly deriving from a third contributor, led to the erroneous exclusion of the scratched male as a possible contributor to the composite autosomal STR profile. Allelic dropouts affecting the female volunteer's profiles further complicated this situation. However, for the majority of the profiles obtained from samples immediately collected after scratches, autosomal STR profiling enhanced Y-STR results by providing very high LR values. Thus, the combined analysis of Y-chromosomal and autosomal STRs is recommendable for real forensic investigations in violence against women cases.

The persistence study confirmed that the (male) cellular material under fingernails is usually lost over time through normal daily activities. Nevertheless, genetic profiles matching the genotypes of the scratched males were obtained even 6, 12 and 24 hours after the scratching. However, when long time lag separated the mock assault and the sample collection, the chances for obtaining genetic profiles arising from normal, non-(mock) crime-related contacts, distinctly increased, which complicated the assessment of the genetic findings.

In general, the findings of this study suggest that in violence against woman casework, the collection of fingernail samples should be performed as soon as possible to increase the chances of obtaining meaningful results. Nevertheless, it is also worth to collect fingernail samples after extended post-assault periods, while bearing in mind that non-assault related DNA transfer(s) might have occurred in the meantime. Particularly, this applies to intimate partners. The interpretation of forensic genetic profiles should always allow for such alternative non-crime related scenario.

Lastly, toward the building of a best practice consensus for forensic fingernail analysis, the recovery study showed that a finger by finger collection should be preferred over the cumulative strategy, as it allows to generate good quality profiles, while avoiding the risk of creating mixtures comprising the genetic material of different contributors found under different fingernails. In addition, as the transfer of genetic material under fingernails is very common and its mechanisms are not completely clarified, the adoption of standard operating procedure for fingernail evidence collection and preservation and the use of sterile disposable tools is imperative in real casework. Moreover, crucial attention has to be paid to the training of practitioners, the establishment of a staff elimination database and the availability of dedicated, constantly monitored sampling areas, equipment, examination areas and specifically trained lab staff. These measures are necessary to control for and avoid potential contamination events that may ultimately lead to miscarriage of justice.

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