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PARVOVIRAL GENOMES IN HUMAN SOFT TISSUES AND BONES OVER DECADES

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ACADEMIC DISSERTATION

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All we have to decide is
what to do with the time that is given us.

Gandalf the Grey
in the Lord of the Rings by J. R. R. Tolkien

CONTENTS

TIIVISTELMÄ

Osa viruksista jättää ensi-infektion jälkeen perintöaineksensa eli genominsa pysyvästi isännän kudoksiin. Tätä kutsutaan persistenssiksi, ja se on usein elinikäistä. Tämän väitöskirjan päätavoitteina oli tutkia parvorokkoviruksen persistenssikykyä, molekyyliepidemiologiaa ja evoluutiota. Parvorokkovirus on pienenpieni ihmisen DNA-virus. Sen on osoitettu persistoivan ihmisen pehmytkudoksissa, mutta persistenssin tautiassosiaatiot ja mekanismit, sekä solu- että molekyylitasolla, ovat vielä tuntemattomia.

Väitöskirjatyön alussa kehitimme kvantitatiivisen polymeraasiketjureaktio (qPCR) -menetelmän, joka tunnistaa, määrittää pitoisuuden ja erottelee kaikki kolme parvorokkoviruksen alatyyppiä. Parvorokkovirusmäärän mittaus on tärkeää ensi-infektoiden ajoittamisessa, persistenssimekanismien selvittämisessä ja viruksen seulonnassa verituotteista.

Seuraavaksi tutkimme parvorokkoviruksen persistenssiä kolmessa eri kudoksessa: nielurisoissa, luuytimessä ja luussa. Virusgenomien esiintyvyys oli korkeaa (35–74 %). Tulokset ovat yhtenäisiä aiempien tutkimustulosten kanssa (nielurisat), osoittivat korkeampaa esiintyvyyttä (luuydin), ja kuvasivat kokonaan uuden kudoslajin, jossa parvorokkovirus persistoi (luu).

Selvitimme nielurisoilla ensimmäisenä maailmassa solutyypin, joka mahdollistaa parvorokkoviruksen persistenssin. Havaitsimme viruksen säilyvän B-soluissa, ja osoitimme, että päästäkseen solun sisään virus hyödyntää elimistömme muodostamia vasta-aineita. On mahdollista, että tämä viruksen vasta-ainevälitteinen kulkeutuminen soluun edistää persistenssin tai oireiden kehittymistä. Lisäksi havaitsimme neljän >45-vuotiaan henkilön B-soluissa parvorokkoviruksen alatyypin 2, jonka tiedetään kadonneen kierrosta 1970-luvulla. Koska parvorokkovirus ei muunnu kudospersistenssin aikana tai kykene uudelleenaktivoitumaan, päättelimme, että nämä henkilöt olivat kantaneet virusta elimistönsä B-soluissa jopa 40 vuotta. Onkin mahdollista, että ihmisen vasta-ainevälitteinen immuunipuolustus perustuu vuosikymmenienkin jälkeen kyseisen patogeenin kohdanneisiin alkuperäisiin B-soluihin.

Tutkimme perimätasolla parvorokkoviruksen taudinaiheuttamiskykyä ja evoluutiota. Määritimme usealta henkilöltä parvorokkoviruksen perintöaineen emäsjärjestyksen. Havaitsimme, että kunkin ihmisen eri kudoksissa esiintyy vain yhtä parvorokkoviruksen alatyyppiä. Vertailimme viruksen perintöaineen muodostamia sekundaarirakenteita. Emme havainneet eroja alatyyppien 1 ja 2 välillä, joten päättelimme, että sekundaarirakenteet eivät selitä tyypin 2 katoamista. Analysoimme

parvorokkoviruksen perintöainesta myös toisen maailmansodan sotavainajien luista. Tämä työ selvensi parvorokkoviruksen esiintyvyyttä noin vuosien 1923-1944 välisenä aikana Pohjolassa ja synnytti aivan uuden tutkimussuuntauksen, arkeovirologian, jossa virusten esiintyvyyttä ja evoluutiota on mahdollista selvittää analysoimalla muinaisia ihmisjäänteitä.

Kuvaammekin tässä väitöskirjassa – ensimmäisenä maailmassa – persistoivia virusgenomeja ihmisen luussa. Selvitimme, parvorokkoviruksen ohella, yhteensä 37 DNA-viruksen esiintyvyyttä nykyihmisten reisiluissa qPCR- ja massiiviparalleelisekvensointimenetelmillä. Löysimme ennennäkemättömän suuren määrän viruslajeja mukaan lukien useita parvo-, herpes-, papillooma- ja polyoomaviruksia, sekä hepatiitti B viruksen ja TT-viruksen.

Tuloksemme kertovat ihmisluiden – sekä tuoreiden että ikivanhojen – soveltuvan erinomaisesti virusten molekyylitason epidemiologian ja evoluution tutkimiseen. Sotavainajatutkimuksemme jälkeen viruslöydöksiä onkin julkaistu jopa tuhansia vuosia sitten eläneiden ihmisten hampaista ja luista. Ihmisluut voivatkin tulevaisuudessa mullistaa virusten ja infektiotautien historian tutkimuksen.

ABSTRACT

Some viruses can establish life-long persistence in their hosts after primary infection. The main aims of this thesis were to investigate the persistence, molecular epidemiology, and evolution of human parvovirus B19 (B19V). B19V is a small single-stranded DNA virus that has been shown to persist in various human soft tissues. The disease associations or mechanisms of this persistence, both at the cellular and molecular level, have remained unknown.

At the beginning of this thesis work, we developed a quantitative PCR (qPCR) method for the detection, quantification, and differentiation of all three B19V genotypes. The method was shown to be suitable for B19V diagnosis and research both in human body fluids and solid tissue samples. Measurement of the B19V load is essential in i) timing of the primary infection, ii) investigation of the persistence mechanisms, and iii) plasma pool screening in blood product manufacturing.

Next, we performed in-depth studies on the B19V persistence in tonsil, bone marrow, and bone. B19V-DNA tissue prevalences were high (35-74 %). Our results were in line with previous publications (tonsils), showed a higher prevalence than earlier detected (bone marrow), and described an entirely new tissue harboring B19V DNA (bone).

We described, for the first time, the cell type accounting for the lifelong B19V-DNA tissue persistence in tonsils. Our results showed that B19V persists preferentially in B cells and that the entry mechanism into these cells is antibody-mediated. Antibody-dependent enhancement (ADE) may account for the B19V symptoms during primary infection or play a role in the establishment of persistence. In B cells of four individuals over 45 years, we detected a B19V genotype 2 that had disappeared from circulation before the 1970s. As B19V does not evolve or reactivate within human tissues, these individuals must have carried the genotype 2 virus in their B cells for over 40 years, suggesting a survival time of several decades for these B cells. Indeed, immunological memory may be, in addition to antigen re-exposure, due to a decades-long survival of the original B cell clones.

In addition to persistence, we investigated in this thesis B19V molecular epidemiology and evolution. We constructed several full-length genomic sequences of B19V genotypes 1 and 2. By combining our bone marrow and bone data, we observed that within an individual, a single B19V genotype is present. We found that the inverted terminal repeat (ITR) sequences, crucial for B19V replication, are similar in both genotypes, and thus, do not explain the disappearance of genotype 2. We searched B19V DNA from long bones of

World War II (WWII) casualties and found an astonishingly high prevalence (45 %). This work, for the first time, shed light on the epidemiological occurrence of B19V in Northern Europe between ~1923 and 1944 and laid the foundation for a new research approach: archeovirology, i.e., the study of virus occurrence and evolution via ancient relics.

Indeed, we describe in this thesis – first in the world – persistent viral genomes in human bone. In addition to B19V, we investigated the prevalences of altogether 37 human DNA viruses in contemporary femoral bones via qPCR and next-generation sequencing (NGS). We discovered several members of the parvo-, herpes-, papilloma- and polyomavirus families, as well as hepatitis B virus and torque teno virus. Thus, our findings revealed bone to be a much richer source of persistent DNA viruses than ever perceived.

Our results show that human skeletal remains – both contemporary and ancient – are suitable material for the investigation of epidemiologies and evolutionary histories of DNA viruses. After our work on WWII bones, archeovirology has expanded: other groups have described B19V, hepatitis B virus, and variola virus sequences in thousands of years old dental and skeletal human remains. Ancient specimens will provide an unforeseen historical perspective on viruses and infectious diseases.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by their roman numerals:

I   **Toppinen M**, Norja P, Aaltonen L-M, Wessberg S, Hedman L, Söderlund-Venermo M, Hedman K. A new quantitative PCR for human parvovirus B19 genotypes. Journal of Virological Methods 2015, 218: 40-45.

II   Pyöriä L*, **Toppinen M***, Mäntylä E, Hedman L, Aaltonen L-M, Vihinen-Ranta M, Ilmarinen T, Söderlund-Venermo M, Hedman K, Perdomo MF. Extinct type of human parvovirus B19 persists in tonsillar B cells. Nature Communications 2017, 8: 14930.

III   **Toppinen M***, Perdomo MF*, Palo JU, Simmonds P, Lycett SJ, Söderlund-Venermo M, Sajantila A, Hedman K. Bones hold the key to DNA virus history and epidemiology. Scientific Reports 2015, 5: 17226.

IV   **Toppinen M**, Pratas D, Väisänen E, Söderlund-Venermo M, Hedman K, Perdomo MF, Sajantila A. The landscape of persistent human DNA viruses in femoral bone. Forensic Science International: Genetics 2020, 48: 102353.

V   **Toppinen M**, Sajantila A, Pratas D, Hedman K, Perdomo MF. The human bone marrow is host to several DNA viruses. Manuscript.

*shared first authorship

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ABBREVIATIONS

A6	B19V genotype 2 strain
B19V	human parvovirus B19
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	Celsius
CPV-2	canine parvovirus type 2
Cq	quantification cycle
D91.1	B19V genotype 3 strain
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
EPC	erythrocyte progenitor cell
FDA	The Food and Drug Administration of the United States
FPV	feline panleukopeniavirus
GT	genotype
HBoV1	human bocavirus 1
HBV	hepatitis B virus
HHV	human herpesvirus
HIV	human immunodeficiency virus
HPD	95 % high probability density interval
HPV	human papillomavirus
HPyV	human polyomavirus
HUSLAB	Helsinki University Hospital Laboratory
ICTV	International Committee on Taxonomy of Viruses
IgG	immunoglobulin G
IgM	immunoglobulin M
ITR	inverted terminal repeat
IU	International Unit
JCPyV	JC polyomavirus
kb	kilo base
kDa	kilo dalton
LaLi	B19V genotype 2 strain
MCPyV	Merkel cell polyomavirus
MPS	massively parallel sequencing
MRCA	most recent common ancestor
NAT	nucleic acid testing
NCBI	National Center for Biotechnology Information of the United States
NGS	next generation sequencing

NIBSC	National Institute for Biological Standards and Control of the United Kingdom
nm	nanometer
NS1	non-structural protein 1
nt	nucleotide
ORF	open reading frame
p(A)d	distal polyadenylation site
p(A)p	proximal polyadenylation site
P6	B19V promoter
PCR	polymerase chain reaction
PMI	post-mortem interval
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNaseP	ribonuclease P
SNP	single nucleotide polymorphism
ssDNA	single-stranded deoxyribonucleic acid
subs	substitutions
TTV	torque teno virus
V9	B19V genotype 3 strain
VLP	virus like particle
VP1	virus protein 1
VP1u	virus protein 1 unique region
VP2	virus protein 2
WHO	World Health Organization
WWII	Second World War

1 INTRODUCTION

Viruses are minute particles consisting of – at the minimum – a genome protected by a protein capsid. Nevertheless, the world of viruses holds enormous diversity. Viruses vary in protein structure, genetic material (RNA or DNA), hosts, and additional features such as lipid envelopes and survival strategies.

Not all animal viruses undergo replication by a lytic cycle[*]. Some viruses can stay latent (i.e., hidden) in their hosts, even for decades. These viruses must have two properties: persistence and reversibility[1]. The latter refers to the capacity of the genome to reactivate from the dormant state to produce infectious progeny virions, a critical requirement of latency. The interplay between the virus's gene expression and host's immune system is crucial in controlling the viral latency, and reactivations generally occur as a result of malfunctions in this equilibrium[2]. The best-known latent viruses are herpesviruses. The diseases caused by reactivations of human herpesviruses are well known even for a non-virologist, for example cold sores (*herpes labialis*) and shingles (*herpes zoster*) caused by Herpes Simplex -virus 1 (HSV1) and Varicella zoster -virus (VZV), respectively[3].

Furthermore, some viral genomes may stay in their host long after primary infection but lack the ability to reactivate. This is termed persistence. The disease associations or even the mechanisms behind viral persistence remain, in most cases, yet to be discovered.

Mutations, the driving force of evolution, shape all organisms, including viruses. As their genomes are haploid (except for retroviruses)[4], alterations in genomic code appear instantly. The viral genomes are either replicated by the host's replication machinery or by their own polymerases[5]. Especially RNA-dependent RNA polymerases are error-prone as they lack the proofreading capacity; thus, the evolution of RNA viruses is considered fast compared to that of DNA viruses[6,7]. Viral evolution studies shed light on the history and pathogenesis of viruses. Evolutionary rates are commonly predicted via computation studies performed with contemporary viral sequences[8].

This doctoral thesis focuses on human parvovirus B19 (B19V). Parvoviruses are non-enveloped viruses with a single-stranded DNA genome. Their name derives from the Latin words "*parvum*" meaning small and "*virus*" meaning

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[*] Lytic cycle = Virus enters and replicates within a host cell to produce new viral particles and eventually causes lysis of the cell.

poison. Parvoviruses are among the smallest viruses with a virion diameter of ~25 nanometers (nm)[9].

B19V belongs to the genus *Erythroparvovirus* in the subfamily *Parvovirinae* of the family *Parvoviridae*[10]. The virus was discovered in 1975 from the plasma of nine asymptomatic blood donors and two patients: one with acute hepatitis and another undergone renal transplantation a week earlier[11]. After B19V primary infection, which generally occurs in childhood, the virus establishes life-long genome persistence in various human organs. So far, the effects of this persistence to the host, both at molecular and cellular levels, have remained unknown. Parvoviruses evolve at a pace approaching that of RNA viruses[9,12]; however, with yet unidentified mechanisms. Thus, this doctoral thesis explores B19V persistence and molecular epidemiology, including evolution. To this end, we also developed the required molecular methodologies.

2 REVIEW OF THE LITERATURE

2.1 PARVOVIRUS B19

2.1.1 GENOME

Parvovirus B19 (B19V) genome is a 5596 nucleotides (nt) long single-stranded linear DNA molecule (Fig. 1a)[13,14]. Identical inverted terminal repeats (ITRs) consisting of 383 nt are located at both ends of the viral genome. Of each ITR, the distal 365 nt fold into two hairpin configurations, referred to as flip and flop[15]. The ITRs contain the origin of replication and are utilized during viral replication[16].

B19V genome encodes five proteins, non-structural protein 1 (NS1), viral proteins 1 and 2 (VP1, VP2), 7.5 kDa protein, and 11 kDa protein, in three open reading frames (ORFs) (Fig. 1b)[17–21]. VP1 and VP2 are encoded by overlapping ORFs and are identical with the addition of 227 amino acids (aa) at the N-terminus of VP1, referred to as the VP1 unique region (VP1u)[17,22]. All viral transcripts are produced from a single promoter, P6[23]. The genome has two polyadenylation sites: the proximal (pA)p is used for Ns1 and 7.5 kDa protein, and the distal (pA)d for VP1/VP2 and the 11 kDa protein[24].

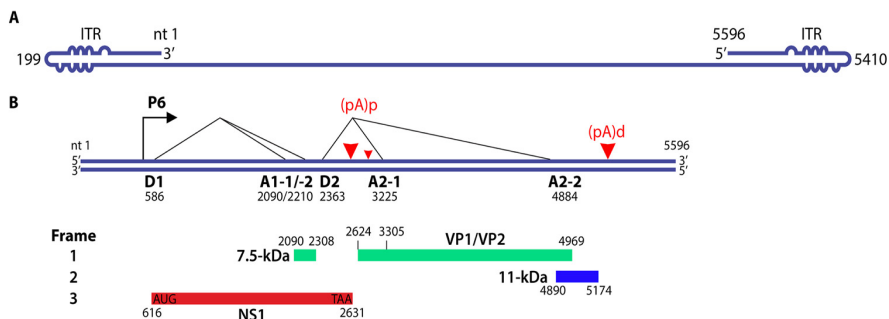**Figure 1**. B19V genome. a) Identical inverted terminal repeats (ITRs) are located at each end of the genome. b) The coding region of 4830 nt encodes five proteins: NS1, VP1, VP2, 7.5kDa, and 11 kDa from a single promoter P6. (pA)p and (pA)d refer to the two polyadenylation sites. Figure reproduced with modifications from Qiu *et al.*[9] with permission from the copyright holder (American Society for Microbiology).

2.1.2 GENOTYPES

Based on the nucleotide sequence, altogether three B19V genotypes have been identified (Fig. 2). The prototype is referred to as genotype 1 (GT1) and was the first virus type found in 1975[11,25]. In 1999 and 2002, two new genotypes were discovered[25–28], and they are referred to as genotypes 2 and 3 (GT2, GT3). Moreover, genotype 3 has two subgenotypes, referred to as genotypes 3A and 3B (GT3A, GT3B), respectively[25,29]. Similarly, for genotype 1, two subgenotypes (1A and 1B) have been proposed[30–34].
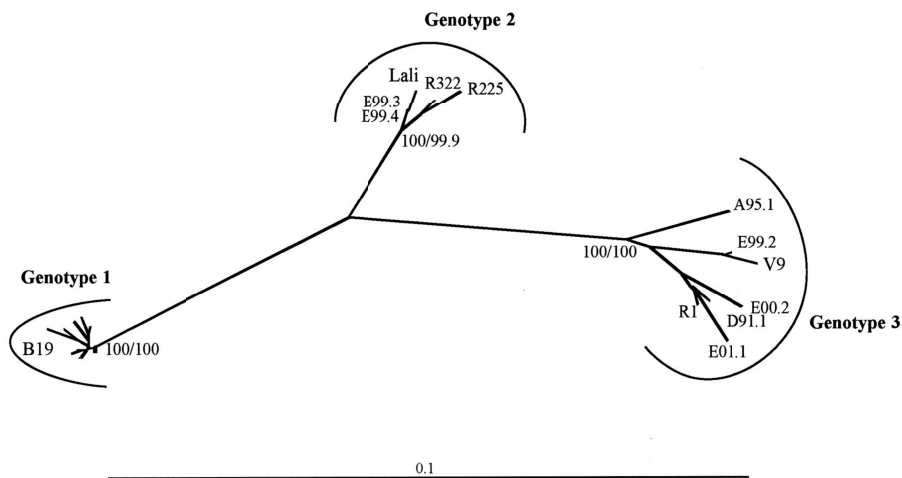
Figure 2. Phylogenetic relationships of B19V genotypes. The analysis was performed with NS1-VP1u nucleotide sequences (994 nt) by using the neighbor-joining algorithm with Kimura 2-parameter. Significant bootstrap values (in percentages) are indicated for 103 neighbor-joining/maximum-parsimony replicates. Figure reproduced from Servant *et al.*[25] with permission from the copyright holder (American Society for Microbiology).

B19V genotypes differ within the coding region by approximately 10 % and in the promoter region by ~20 % when compared at the nucleotide level[25,28]. At the amino acid level, the difference is ~1-6 % with majority of the substitutions being synonymous[35]. The B19V genotypes do not differ by their antigenic properties: immunological cross-reactivity has been demonstrated, and all three form a single serotype[35,36]. Genotype-specific diseases have not been described. Co-occurrences of two genotypes within an individual are rare[37–39].

The ITR sequences of genotype 1 have been analyzed in detail. Only one full-length genotype 2 sequence with ITRs has been published[40] (GenBank

accession number AB550331). Recently, a preprint appeared in bioRxiv presenting sequencing of ancient full-length genotype 3 strains[41], however, the sequences are not yet available in Genbank.

Spatiotemporal differences between the B19V genotypes have been described. Nowadays, genotype 1 is the main virus type circulating and giving rise to primary infections globally[42]. Genotype 2 has been detected mainly in tissue samples of individuals born before the 1970s[43–45]. Only a few cases of B19V genotype 2 in plasma/serum have been reported (Table 1), mostly in immunocompromised individuals.

B19V genotype 3 has been found mainly in Brazil, Ghana, and India (Table 2); however, the most common genotype in these countries is, as elsewhere in the world, genotype 1. Only a few sporadic findings of genotype 3 in Europe exist, including the original detections of V9-like (GT3A) and D91.1-like (GT3B) viruses in France[25,26,46]. The original genotype 3A was found in a 6-year-old male child who had lived in France since birth[26]. Interestingly, Schneider *et al.*[37] investigating livers of individuals living in Germany reported that all the study subjects carrying B19V genotype 3 originated from abroad (Morocco, Turkey, Egypt) and concluded that these individuals might have received the genotype 3 infection outside of Germany.

Table 1. B19V genotype 2 findings in plasma/serum samples.

Study	Genotype 2 findings
Nguyen *et al.* 2002[27]	The GT2 strain A6: serum from an anemic Italian HIV patient (sample collected 1991-1998).
Schneider *et al.* 2004[47]	Two plasma-derived coagulation factor concentrates from 2000-2003 (S/D & dry heat 100 °C 0.5 h). Three (non-inactivated) Factor VIII concentrates, administrated until the beginning of 1980s.
Blümel *et al.* 2005[36]	A high-titer contaminant in donor plasma in Germany (no information of collection time).
Liefeldt *et al.* 2005[48]	Serum of a 34-year-old renal transplant patient (collected in 2001). The recipient's sera prior to and early after transplantation were B19V-DNA negative as well as VP1/VP2-IgM and IgG negative. The donor serum was IgG positive, but IgM and DNA negative. At three months after transplantation, B19V load in the recipient's serum was ~1E11 copies/ml.
Koppelman *et al.* 2007[49]	A blood donation in the Netherlands (sample collected 2005-2007).
Corcoran *et al.* 2010[50]	Three cases in South Africa (samples collected 2006-2008): serum from an HIV-infected 11-year-old child with anemia; and two cases with no medical records.
Grabarczyk *et al.* 2011[51]	Two immunocompromised patients in Poland: A 29-year-old female, received in 2003 a kidney transplant from a 46-year-old woman; An 18-year-old female, diagnosed with T-lymphoblastic leukemia in 2008.
Tsujikawa *et al.* 2012[40]	A donor plasma in the United States (no information of collection time); a full-length clone obtained
Eis-Hübinger *et al.* 2014[52]	Two blood donations in Germany (samples collected 2006-2008).
Ivanova *et al.* 2016[53]	A Bulgarian female patient born in the early 1970s, no information about recent travel, transfusion, immunosuppression, or transplantation history (sample collected 2009)

GT2=genotype 2

HIV=human immunodeficiency virus

S/D=solvent/detergent

VP1/VP2=viral protein 1/viral protein 2

IgM=immunoglobulin M

IgG=immunoglobulin G

Table 2. Worldwide findings of B19V genotype 3 in plasma/serum samples.

Country	Study	Samples			Medical status	B19V DNA+ cases		
		Collection period	Type(s)	N=		GT1+	GT2+	GT3+
Brazil	Freitas et al. 2008[54]	1995-2005	serum	487	symptomatic individuals (suggestive of B19V infection)	106	0	11
	Garcia et al. 2017[55]	1996-2006	serum	32	patients with erythema infectiosum and HIV patients testing positive for B19V DNA	31	0	1
	Alves et al. 2020[56]	2004-2012	serum	30	patients with acute liver failure undergoing liver transplantation	3	0	4
Ghana	Candotti et al. 2004[57]	1999-2001	plasma	1000	healthy blood donors	0	0	12
	Parsyan et al. 2006[58]	2003-2005	plasma	700	blood donors or children receiving transfusion (pre-transfusion sample)	0	0	26
	Parsyan et al. 2007[29]	2002-2004	plasma	7	GT3+ blood donors or children receiving transfusion (pre-transfusion sample) in study[58]	0	0	7
India	Jain et al. 2013[59]	unknown	serum	2	a case report of two children with acute left ventricular dysfunction	0	0	2
	Jain et al. 2015	2011-2012	serum	238	children with hematological malignancies	11	0	2
	Jain et al. 2016[60]	2013-2015	serum	96	children receiving chemotherapy for solid malignancies	0	0	21
	Jain et al. 2018[61]	2014-2015	serum	233	children with hematological malignancies	0	0	38
South Africa	Corcoran et al. 2010[50]	2006-2008	serum	239	immunocompromised patients suspected of having chronic B19V infection	40	2	10

Country	Study	Samples			Medical status	B19V DNA+ cases		
		Collection period	Type(s)	N=		GT1+	GT2+	GT3+
France	Nguyen et al. 1999[26]	1995	serum	1	case report of a 6-year-old child with headache and dysuria, the original GT3A-finding	0	0	1
	Servant et al. 2002[25]	1992-2001	serum	741	HIV-infected adults with chronic anemia, clinical samples of suspected B19V infection, the original GT3B-finding	165	0	11
USA	Rinckel et al. 2009[46]	unknown	plasma	81 000	blood donors	0	0	1
Turkey	Audin et al. 2019[33]	2009-2016	serum	12	diagnosed B19V+ cases of children and adults	10	0	2
Burkina Faso				5		0	0	5
Nigeria	Hübschen et al. 2009[42]	2000-2008	serum	5	rash/fever patients negative for measles and rubella, and thus, studied for B19V infection	2	0	3
Kyrgyzstan				24		20	0	4
Greece				21		20	0	1

+= positive
GT=genotype
HIV=human immunodeficiency virus

2.1.3 TRANSMISSION, EPIDEMIOLOGY, AND DISEASES

B19V infection can be transmitted via three routes: respiratory droplets, vertical transmission from mother to fetus, and blood products[62–67]. Most B19V infections are acquired before age 10. During primary infection, viremia with up to 1E14 copies/ml of blood can be demonstrated[68]. The viremic peak rapidly decreases; however, the DNA may be detected in blood for several months up to a year[69–71] (Fig. 3). Two recent studies suggested that the residual low-level B19V DNA in blood would be naked DNA; i.e., DNAemia as opposed to viremia[72,73].

Following B19V infection, a humoral response is elicited with B19V-specific immunoglobulin M (IgM) and G (IgG) antibodies[62,74,75]. The occurrence of B19V DNA and antibodies (IgM, IgG, and IgG avidity, i.e., the binding force between the antigen and antibody[76]) in serum after the infection is presented in Figure 3. IgM antibodies are produced first and persist in serum for a few months, while IgG antibodies, which are produced later, presumably persist for life and protect against secondary infections[62]. The prevalence of anti-B19V IgG increases with age: ~2-20 % in children <5 years, ~40-60 % in adolescents and young adults, and ~60-80 % in the elderly (>65 y)[77–79].

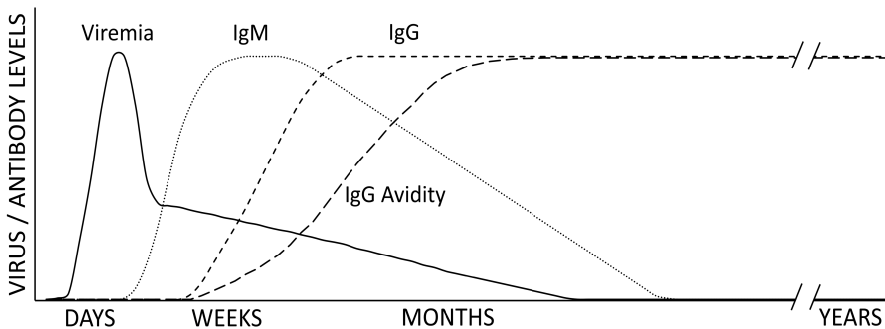**Figure 3**. B19V DNA (viremia) and antibodies (IgM and IgG) in human serum after a primary infection.

In most cases, B19V primary infections are asymptomatic or with mild symptoms, resembling those of a common cold[80,81]. The manifestations may include arthralgia and *erythema infectiosum* (fifth disease)[62,82]. Maternal primary infection during pregnancy can have severe complications: vertical

transmission within the first and early second trimester can lead to hydrops fetalis or even fetal death[64,83].

Asymptomatic primary infection with a high B19V load constitutes a risk in blood donation. Several studies of B19V in blood products have been conducted[47,84–87], both before and after the era of implementation of inactivation procedures in plasma product and clotting factor concentrate manufacturing. Indeed, the minute (25 nm) non-enveloped virus capsid is extremely resistant to most virus inactivation procedures. Thus, the only way is to monitor the B19V load in plasma pools (mini- or maxipools) by quantitative PCR. The Food and Drug Administration of the United States (FDA) and the European Directorate for the Quality of Medicines and Healthcare (EDQM, Council of Europe) have set a limit for B19V load in blood donations[88]. Each donation with B19V-DNA level above 1E4 international units /ml (IU/ml) of plasma is withdrawn. B19V loads below the limit are not thought to lead to seroconversion[88,89]. Nonetheless, Soucie *et al.*[90] found 1.7 times higher B19V-IgG seroconversion rate in children having received low-level B19V plasma products than those having received recombinant products.

2.1.4   CELL TROPISM AND ENTRY

In B19V infection, the erythroid progenitor cells (EPCs) of human bone marrow are the primary target and the main replication site of B19V[91,92]. The erythrocyte P antigen (globoside), expressed on the surface of EPCs, has long been considered as the primary B19V receptor[93]. Individuals who lack globoside are resistant to B19V infection[94]. Fetal cardiac myocytes, which express globoside, are permissive for the virus, while myocytes of older children and adults lacking the globoside are non-permissive[95–97]. However, not all globoside expressing cells are permissive for the virus[98].

Recently Bieri and Ros[99] showed with globoside knock-out human megakaryoblastoid UT7/Epo cells that while globoside is essential for productive infection, it is not required for B19V entry. Initially, alpha5beta1 integrin and Ku80 autoantigen were proposed as B19V co-receptors[100,101]. Moreover, based on studies on the VP1u region, an unidentified co-receptor binding the VP1u has been hypothesized[102,103]. Upon B19V uptake into EPCs, globoside binding to capsid protein VP2 induces structural changes in the virion exposing the N-terminal part of VP1u, allowing for internalization[104,105].

Moreover, B19V has been shown to use antibody-dependent enhancement (ADE) in viral uptake[106,107]. In ADE, virus-specific antibodies interact with both i) the host cell and ii) the virus capsid, leading to enhanced entry and in some cases enhanced replication of the virus[108]. Most commonly, the

enhanced uptake occurs via the Fc-portion of IgG and Fc-receptors on the cell surface. Additionally, a complement-mediated route has been described (Fig. 4). Both of these mechanisms have been shown to occur with B19V: the Fc-receptor mediated ADE with monocytes and the complement-mediated with endothelial cells[106,107]. ADE operates with a variety of viruses including the Aleutian mink parvovirus[108,109]. ADE in a viral infection may lead to exacerbated illness (e.g., with Dengue virus and SARS coronavirus); and in vaccine development, particular attention is required for achieving a vaccine that induces protective immunity.
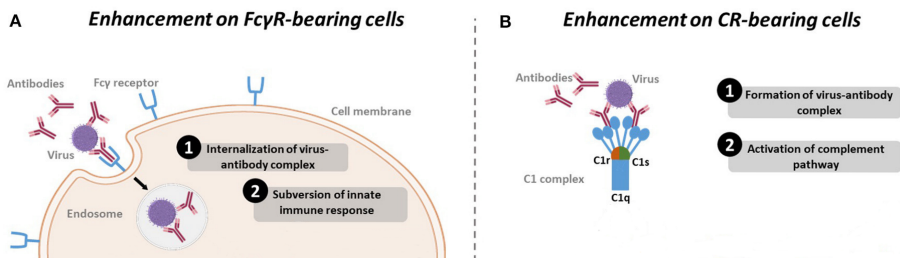

Figure 4. Mechanisms of antibody-dependent enhancement (ADE). A) Fcγ-receptor (FcγR) mediated, and B) complement receptor (CR) -mediated ADE. Figure reproduced with modifications from Smatti *et al.*[110].

2.1.5   PERSISTENCE IN HUMAN TISSUES

After primary infection, B19V establishes lifelong genome persistence in various human tissues. The cellular and molecular mechanisms of B19V-DNA persistence are unknown. Nevertheless, over 60 publications have documented the presence of B19V DNA in a large variety of human soft tissues including salivary gland[111], thyroid[112–114], liver[37,43,115–117], spleen/lymph nodes[44], kidney[118,119], bone marrow[29,38,44,120,121], muscle[122], synovia[38,43,123–128], skin[28,38,43,129–133], testis[134–138], heart[38,45,139–149], tonsils[43,126,150] and brain[44,151–153] (Fig. 5). In approximately half of the studies, serological analyses were performed. In those, individuals with persistent B19V DNA in their organs showed past immunity (i.e., B19V-IgG positivity and IgM negativity).

The B19V-DNA prevalences in human organs are high, with some variation in organ types (Fig. 5). In general, over 50 % DNA prevalences have been reported for most organs (of grown-ups). Only salivary glands, muscle, and

tonsils have shown lower prevalences, albeit in rare studies. The methods used in the 1990s and early 2000s might have failed to detect all B19V types. Indeed, when Hokynar *et al.*[28] examining human skin discovered B19V genotype 2, they also showed that the ordinary methods of the time failed in detection of this genotype. The prevalence in human skin of genotype 2 was 47 %, as opposed to 26 % of genotype 1.
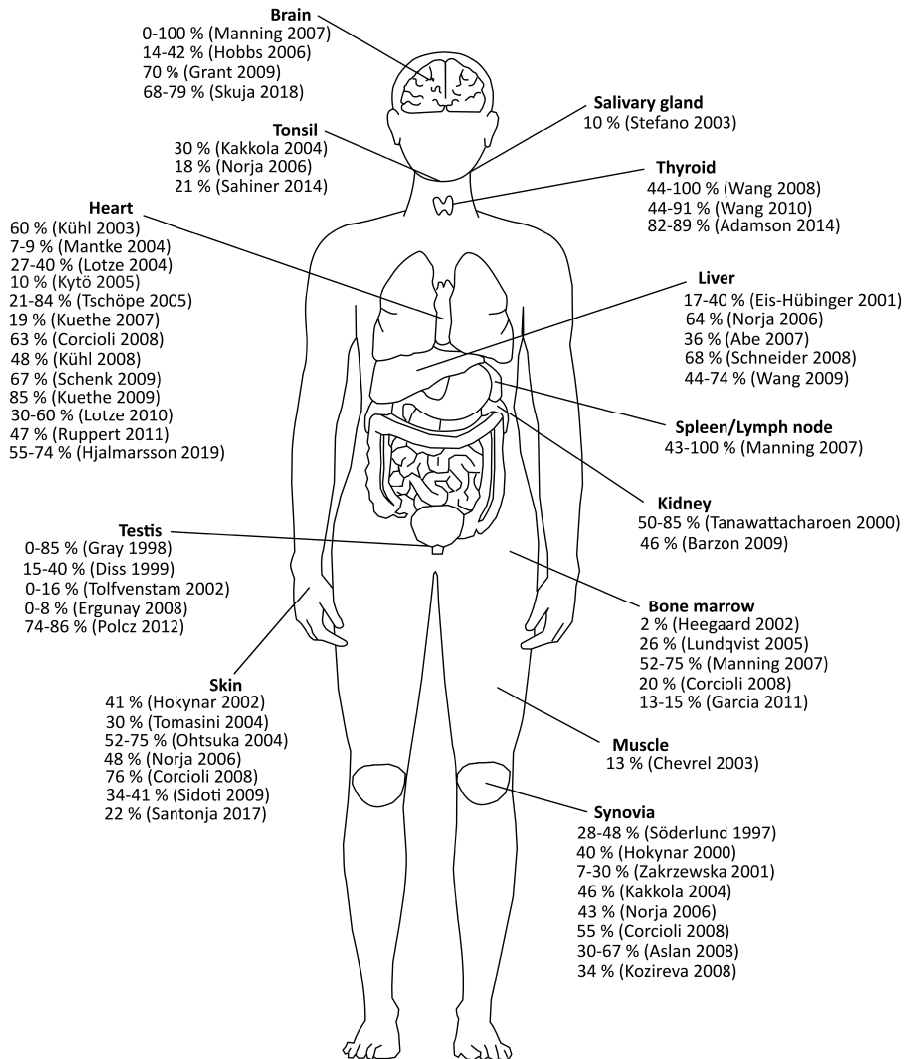
Brain
0-100 % (Manning 2007)
14-42 % (Hobbs 2006)
70 % (Grant 2009)
68-79 % (Skuja 2018)

Salivary gland
10 % (Stefano 2003)

Tonsil
30 % (Kakkola 2004)
18 % (Norja 2006)
21 % (Sahiner 2014)

Thyroid
44-100 % (Wang 2008)
44-91 % (Wang 2010)
82-89 % (Adamson 2014)

Heart
60 % (Kühl 2003)
7-9 % (Mantke 2004)
27-40 % (Lotze 2004)
10 % (Kytö 2005)
21-84 % (Tschöpe 2005)
19 % (Kuethe 2007)
63 % (Corcioli 2008)
48 % (Kühl 2008)
67 % (Schenk 2009)
85 % (Kuethe 2009)
30-60 % (Lotze 2010)
47 % (Ruppert 2011)
55-74 % (Hjalmarsson 2019)

Liver
17-40 % (Eis-Hübinger 2001)
64 % (Norja 2006)
36 % (Abe 2007)
68 % (Schneider 2008)
44-74 % (Wang 2009)

Spleen/Lymph node
43-100 % (Manning 2007)

Kidney
50-85 % (Tanawattacharoen 2000)
46 % (Barzon 2009)

Testis
0-85 % (Gray 1998)
15-40 % (Diss 1999)
0-16 % (Tolfvenstam 2002)
0-8 % (Ergunay 2008)
74-86 % (Polcz 2012)

Bone marrow
2 % (Heegaard 2002)
26 % (Lundqvist 2005)
52-75 % (Manning 2007)
20 % (Corcioli 2008)
13-15 % (Garcia 2011)

Muscle
13 % (Chevrel 2003)

Skin
41 % (Hokynar 2002)
30 % (Tomasini 2004)
52-75 % (Ohtsuka 2004)
48 % (Norja 2006)
76 % (Corcioli 2008)
34-41 % (Sidoti 2009)
22 % (Santonja 2017)

Synovia
28-48 % (Söderlund 1997)
40 % (Hokynar 2000)
7-30 % (Zakrzewska 2001)
46 % (Kakkola 2004)
43 % (Norja 2006)
55 % (Corcioli 2008)
30-67 % (Aslan 2008)
34 % (Kozireva 2008)

Figure 5. B19V-DNA persistence in human tissues. The percentages correspond to DNA prevalences, followed by the first author and publication year. The corresponding references are[28, 29, 37, 38, 43-45, 111-153]

For B19V-DNA long-term persistence, clinical associations have been proposed, especially with synovia and heart. Thus, these organs, as well as skin, are the most studied for B19V DNA. As B19V can cause arthritis shortly after primary infection[154,155], the DNA persistence in synovia has been a popular topic as regards the etiology of rheumatoid arthritis. Similarly, the role of B19V-DNA persistence in myocarditis and dilated cardiomyopathy has been under intensive studies. Indeed, B19V-DNA persists ubiquitously in healthy synovia and heart, but no convincing correlation between diseases affecting these tissues and B19V genome persistence has been substantiated[43,139,141,146,149,156–158].

2.1.6 EVOLUTION

One of the few well-documented evolutionary events of parvoviruses is the adaptation of carnivore parvoviruses from cats to dogs. First, canine parvovirus type 2 (CPV-2) evolved from feline panleukopenia virus (FPV). While FPV was only able to infect cats, the new form CPV-2 did not, but instead dogs. Later, yet another variant, CPV-2a, emerged and replaced the CPV-2 worldwide[159,160]. CPV-2a was found to be able to infect both cats and dogs[161].

The evolution of parvoviruses has been considered rapid, with FPV and CPV-2 having substitution rates of 9.4E-5 and 1.7E-4 subs/site/year, respectively[162,163]. This resembles the rates of RNA-virus evolution (1E-2 to 1E-5 subs/site/year); while dsDNA viruses, such as human herpes- and papillomaviruses, show rates (1E-8 to 1E-9 subs/site/year) approaching those of their host species[8]. Like parvoviruses, other ssDNA viruses show great diversity and fast evolution[8].

The latest evolutionary rate of B19V has been investigated by Mühlemann *et al.*[12] . This study involved 10 B19V genomes from dental and skeletal remains of individuals who lived in Eurasia and Greenland 500 to 6900 years ago, providing resolution for the estimation of B19V evolution. They acquired a substitution rate of 1.2E-5 subs/site/year, which is almost a log higher than previously expected. Furthermore, they estimated the time to the most recent common ancestor (MRCA) of B19V genotypes. The MRCA of all B19V genotypes was placed to 12600 years ago, showing evidence that B19V has been associated with humans for thousands of years[12].

2.2 OTHER PERSISTENT HUMAN DNA VIRUSES

In addition to B19V, other parvoviruses can establish tissue persistence. Altogether nine human parvovirus species have been recognized by the International Committee on Taxonomy of Viruses (ICTV): B19V, adeno-associated viruses (AAV), human bocaviruses (HBoV), parvovirus 4 (PARV4), cutavirus (CuV), tusavirus (TuV), and bufavirus (BuV). AAV was discovered in 1965, and HBoVs, PARV4, CuV, TuV, and BuV after 2005. HBoV1 causes respiratory symptoms, and its DNA persists up to several months after primary infection in respiratory airways[164]. The recently found CuV was detected in the skin of healthy individuals and those suffering of cutaneous T cell lymphoma[165]. PARV4 DNA has been detected mainly in tissues of intravenous drug users[44,166]. In vitro cell culture of human parvoviruses is challenging due to deficient knowledge of the host cell type and its culture conditions.

Other human DNA viruses known to establish persistence or latency are herpesviruses, polyomaviruses, papillomaviruses, and hepatitis B virus. Moreover, anelloviruses are frequently detected in human blood and, thus, in tissues. The properties of each virus family are presented in Table 3.

Table 3. Properties of persistent human DNA viruses.

Family	number of human species*	Capsid size (nm)	Genome size (kb)	Genome organization
Parvoviridae	9^	25	4-6	linear ssDNA
Herpesviridae	9	125-130	125-240	linear (during latency circular) dsDNA
Polyomaviridae	14^	40-45	4.7-5.3	circular dsDNA
Papillomaviridae	49	52-55	8	circular dsDNA
Hepadnaviridae	1	32-36	3.2	circular, partially dsDNA
Anelloviridae	56	30	2-3.9	circular ssDNA

* according to the International Committee on Taxonomy of Viruses (ICTV) 2019 release
^ some species of *Parvoviridae* and *Polyomaviridae* await verification of human as host
nm=nano meter
kb=kilo base
ssDNA=single stranded deoxyribonucleic acid
dsDNA=double stranded deoxyribonucleic acid

Human herpesviruses (HHV) are a classic example of latency, able to reactivate and cause secondary disease. Notable HHVs are Herpes Simplex -viruses 1 and 2 (HSV1 and 2), Epstein-Barr virus (EBV), and Varicella zoster -virus (VZV). While the seroprevalences of HHVs vary, 100 % of the adult human population is seropositive to at least one herpesvirus. The highest prevalence is of EBV (>90 %) and the lowest of Kaposi sarcoma herpesvirus (0-5 %)[167,168]. Among the diseases caused by HSV reactivations are cold sores (*herpes labialis*) and by VZV, shingles (*herpes zoster*)[3]. HHV reactivations can occur even in immunocompetent individuals, exacerbated by environmental or internal factors. The latency sites of some HHVs are known e.g., trigeminal ganglia for HSV1 and VZV and memory B cells for EBV. Latent HHV genomes have, in most cases, a nucleosomal structure similar to that of cellular chromatin[169,170]. However, HHV6 may integrate into the host genome telomers[171]. Approximately 1 % of world's population harbor chromosomally-integrated HHV6 (iciHHV6) that can be inherited in a Mendelian manner, with a 50 % chance of being passed to a child[172,173].

The family *Polyomaviridae* has expanded considerably in recent years with 12 new human polyomaviruses (HPyVs). The classical HPyVs discovered in the 1970s are BK polyomavirus (BKPyV or HPyV1) and JC polyomavirus (JCPyV or HPyV2). A recent study of healthy adult Dutch population showed 60-100 % seroprevalence for all HPyVs except HPyV9, 11, and 12 (4, 5, and 19 %, respectively)[174]. HPyVs establish latency, and life-threatening diseases, such as BKPyV-related nephropathy[175] and JCPyV-related brain disease, progressive multifocal leukoencephalopathy (PML[176]), result from viral reactivation in immunocompromised patients. Merkel cell polyomavirus (MCPyV) causes 80 % of Merkel cell carcinoma (MCC), a rare skin cancer of aged or immune-suppressed individuals[177]. In these cases, MCPyV integrates into the host genome and undergoes mutations[177,178]. Similarly, JCPyV genomic rearrangements occur in PML[179]. A latency site for JCPyV and BKPyV is urinary tract, and for MCPyV, skin. Furthermore, tonsils have been shown to be an HPyV latency site[180].

Human papillomaviruses (HPV) cause persistent infections of skin and mucosae. Infections may be asymptomatic, and usually, the host's immune system clears the infection within a few years. The worldwide prevalence of HPV infection in women without cervical abnormalities is 11–12 %[181]. HPVs are divided into low- and high-risk types; the latter can produce cancers, including cervical and other anogenital, as well as head and neck. HPV-related cancers are among the most common malignancies in humans. For an interested reader, references[181,182] are suggested for an in-depth review of HPV-related diseases.

Hepatitis B virus (HBV) is the only human virus of the family *Hepadnaviridae*. HBV is a major cause of human hepatitis. It may establish chronic infection,

especially if obtained perinatally. In the Western Pacific and Africa, where HBV prevalence is highest (>6 %), infection in infancy or early childhood leads to chronic hepatitis in about 95 % of cases and is a significant public health issue[183]. Among the long-term complications of HBV infections are cirrhosis and hepatocellular carcinoma, a rapidly progressing cancer of the liver.

Anelloviruses are an extremely diverse family of viruses infecting humans and other animals. No disease among humans is known to be related to anelloviruses. The most studied is torque teno virus (TTV). Seroprevalence studies are rare, but IgG reactivities have been observed either towards single or multiple strains pointing to an antigenic distinction of TTV species[184]. Indeed, humans have continuous and recurrent TTV infections, even with several strains at a time[185]. TTV DNA can be detected in healthy individuals' blood at prevalences of 50-90 %[186].

2.3 VIRAL NUCLEIC ACID TESTING

Viral nucleic acid testing (NAT) is a commonly used methodology in infectious disease diagnostics and research. Furthermore, in the blood manufacturing process, NAT is utilized for the detection of microbial pathogens, especially viruses, in blood donations. NAT approaches can detect minute amounts of nucleic acids in a wide range of samples. Qualitative NAT methods show the presence (or absence) of target nucleic acids, while quantitative methods provide the copy numbers of the target present in a sample.

With NAT only the viral nucleic acids are discerned, thus there is no indication on whether whole virus is present. With most viruses, the presence of a viral genome in a clinical specimen is proof of infection. For example, in coronavirus diagnostics, the demonstration of coronavirus RNA within the total nucleic acids isolated from a nasopharyngeal swab is indicative of infection but not necessarily contagiousness[187].

2.3.1 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is currently the most common means of NAT in both virus diagnosis and research. The sensitivity and specificity of a PCR assay are its most relevant features. In principle, a single target molecule present in a sample can be detected. Thus, strict precautions are required to

avoid contamination and false-positive results. Similarly, the assay's inherent specificity for the target needs to be confirmed.

PCR is a nucleic acid amplification technique where the target DNA is amplified via thermostable DNA polymerase and short target-specific oligonucleotides called primers[188]. The amplified DNA can be detected via i) gel-electrophoresis or ii) fluorophores, either DNA binding dyes (e.g., SYBR Green) or additional oligonucleotides called hydrolysis probes (Fig. 6). Hydrolysis probes utilize Förster resonance energy transfer (FRET), and these methods offer usually better specificity than DNA-binding dye-based assays because of the need for a third oligonucleotide to bind specifically the synthesizing DNA. The DNA-binding dyes detect all dsDNA, including primer-dimers and unspecific amplification products.
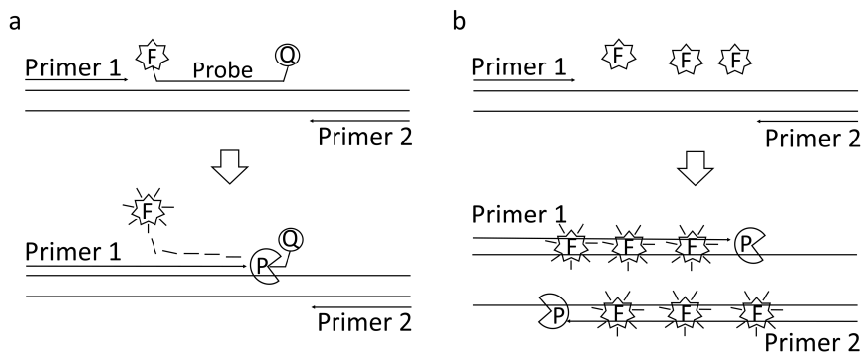

Figure 6. Two principles of DNA quantification by PCR. a) In hydrolysis probe-based qPCR, the fluorophore (F) is quenched until released from the probe by the exonuclease activity of the DNA polymerase (P). b) DNA-binding dye-based methods utilize fluorophores (F) that emit light when bound to synthesizing dsDNA.

Nowadays, quantitative real-time PCR (qPCR) techniques from which the copy number of the target can be deduced are widely used. With modern qPCR machines, up to five targets can be multiplexed, i.e., analyzed simultaneously in one reaction. The quantification is based on comparison to known quantities of the target DNA. These standards are analyzed together with the unknown study specimen and form a standard curve. For example, a ten-fold plasmid dilution series can be used. Furthermore, the World Health Organization (WHO) has been providing, since 1997, international standards (IS) for the calibration of in-house NAT methods[189].

2.3.2 SEQUENCING TECHNIQUES

For decades, the automated Sanger-sequencing method has been the primary viral DNA sequencing technology[190]. Mainly because of its limitations in whole human genome sequencing, new applications emerged in the early 2000s[191]. Now, these next-generation sequencing (NGS, or massively parallel sequencing, MPS) techniques are widely used in human genome analysis and increasingly in virus research.

Sequencing techniques are complementary to qPCR-methods. They are utilized in viral genotyping, investigation of viral evolution, and analysis of mutations, such as single nucleotide polymorphisms (SNPs) or genomic rearrangements. Importantly, novel viruses can be discovered with NGS.

The purpose of all sequencing techniques is the characterization of the nucleotide composition of the DNA. Sanger sequencing yields a single (the most abundant) sequence present in a reaction, and the data analysis is straightforward. In NGS, hundreds of thousands (short) reads are sequenced simultaneously, and the downstream analysis requires a comprehensive package of bioinformatics tools (pipeline, Fig. 7) and computing power.

Next generation sequencing data analysis:

RAW READS

QUALITY CONTROLS

ALIGNMENT-BASED ASSEMBLY
Alignment programs
e.g. Bowtie2

BLASTn

DE NOVO ASSEMBLY
De novo programs
e.g. metaSPAdes

BLASTx

METAGENOMIC COMPOSITION
Taxonomic classification
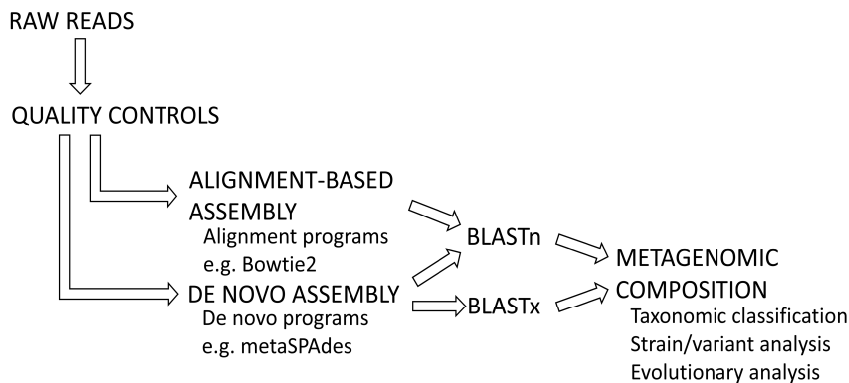Strain/variant analysis
Evolutionary analysis

Figure 7. Essential steps of next generation sequencing (NGS) data analysis, i.e. a pipeline. BLAST = basic local alignment search tool, BLASTn searches nucleotide sequences from nucleotide database, BLASTx searches translated nucleotide products from protein sequence database. Some virus pipelines deplete the human DNA after the quality controls, and not all pipelines do both alignment-based and *De novo* assembly. Moreover, visualization programs and data curation approaches are needed.

All NGS techniques require before the sequencing, tedious sample preprocessing, known as library preparation. This process can include molecular biology tools such as nuclease-treatments, ligations, reverse transcription, and nucleic acid amplification. All library preparation methods ligate the DNA stretches present in the sample with known oligonucleotides, called adapters. These adapters are utilized in amplification of the library as well as in the sequencing process. Amplification of the target DNA is usually required to generate an appropriate amount of DNA containing the adapter sequences; however, also amplification-free library preparation methods do exist[192,193]. The basic principle of a library preparation is presented in Figure 8a.

A fundamental challenge in the analysis of virus DNA is not only the sample quality but also – and rather – the quantity. The presence of human DNA in a sample of tissue or environmental bacteria e.g., in waste water, prevails over the viral DNA. Viral DNA can be exceeded, even over 1000-fold by the nuclear DNA alone. Therefore, amplification alone is not sufficient, but specific enrichment techniques may be needed. The enrichment methods can target i) viral capsids via, for example, ultracentrifugation or endonuclease treatment, or ii) specific (viral) DNA strands via hybridization (Fig. 8b)[194,195]. In the latter, the library is incubated with biotinylated baits, and the virus sequences are captured and enriched from other DNAs.
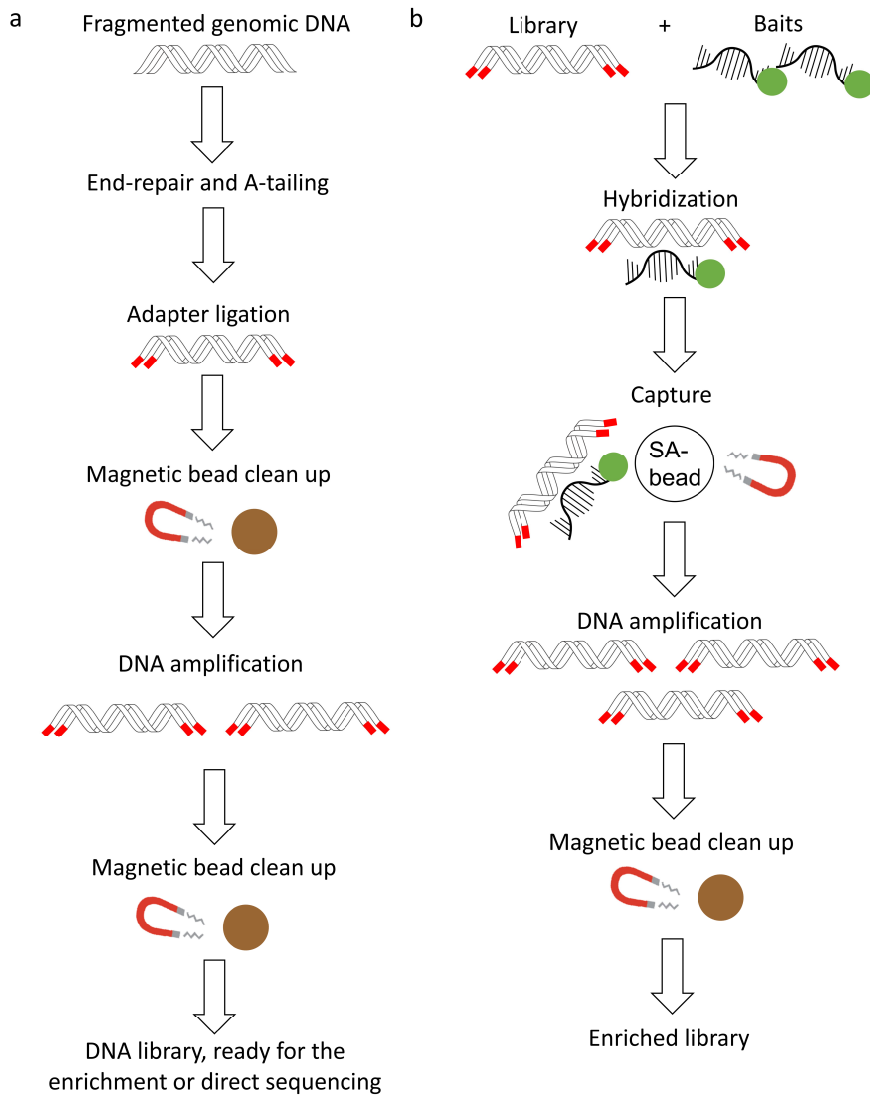
Figure 8. Sample preparation for NGS. a) The library preparation usually starts with sample DNA fragmentation. Then, the fragments are ligated to known oligonucleotides (adapters). b) For targeted enrichment of viruses, the library is incubated with biotinylated baits designed to hybridize with the viruses of interest. Virus sequences are captured and enriched from other DNAs. SA-bead = streptavidin-coated magnetic bead.

2.3.3 PCR OR NGS?

The currently used viral NATs rely heavily on PCR technology. PCR methods require a target pathogen, and only a limited number of pathogens can be analyzed simultaneously (maximum of five with most qPCR instruments). A qPCR result is obtained within 1-3 hours, and the cost per sample, when performed in-house, is a few euros[†]. With NGS-based NAT methods, the sequencing is unbiased and hundreds of unknown genomes can be analyzed simultaneously in a single test. When lacking obvious candidates to test, NGS can provide novel pathogen insights. Yet, NGS-based methods are more expensive, only qualitative, and more time and labor-consuming. In the future, faster NGS sample preparations and user-friendlier methods of data analysis will likely facilitate the implementation of NGS-based techniques in virus diagnostics.

[†] Cost per sample of an in-house qPCR assay is estimated based on commonly available commercial reagents (used in our lab) and includes a qPCR master mix, oligonucleotides, and plastics, no labor.

3 AIMS

The specific aims of this thesis were:

1. to develop and evaluate molecular assays, such as quantitative PCR and next generation sequencing, for the persistent DNA viruses, including parvovirus B19 (B19V),

2. to determine the genomic prevalences and copy numbers of persistent DNA viruses in human soft and hard tissues such as tonsils, bone marrow, and bone,

3. to investigate the utility of human bones for virus research and evolutionary analysis,

4. to define the cell type hosting B19V tissue persistence and to determine the entry mechanism of B19V into those cells,

5. to examine the molecular epidemiology of B19V, including evolution, from the early 1900s to contemporary times

4 MATERIALS AND METHODS

4.1 STUDY SAMPLES

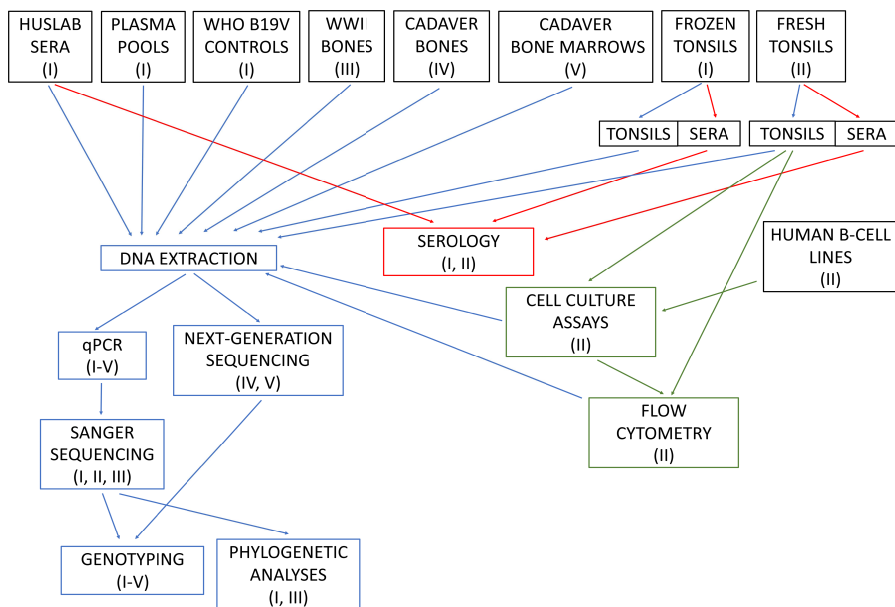Figure 9 flow chart shows the summary of samples and methods used in Studies I-V.

Figure 9. Flow chart of the samples and methods used in this thesis. Blue lines represent DNA work, red lines serology, and green cell culture work. The roman numerals in parenthesis refer to Studies I-V.

4.1.1 SERUM AND PLASMA SAMPLES (I)

Serum samples of Study I were obtained from patients with suspected B19V primary infection and sent to Helsinki University Hospital Laboratory (HUSLAB) for serological studies. The cohort consisted of serum samples from

20 B19V IgM-positive, eight IgM-negative but IgG-positive, and 12 IgM- and IgG-negative subjects. These are referred to as HUSLAB sera throughout the thesis.

WHO International Reference Panel for Parvovirus B19 Genotypes[196] was obtained from the National Institute for Biological Standards and Control (NIBSC). The panel contained one B19V-DNA negative and three B19V-DNA positive (genotype 1, 2, and 3A-like viruses) plasma samples serving as DNA controls for each genotype. The positive samples contained B19V DNA at ~1.00E6 IU/ml plasma[196].

Plasma minipools (N=208) of which each included plasma from 16 individual blood donations were obtained from the Finnish Red Cross Blood Service. Minipools had been tested for B19V DNA and hepatitis A virus (HAV) RNA at the Finnish Red Cross Blood Service (Procleix Parvo/HAV Assay, Gen-Probe and Novartis Diagnostics), as part of routine virus screening. All minipools except one had B19V-DNA values below the Finnish Red Cross Blood Service cut-off. This minipool had B19V DNA at 7.36E2 IU/ml plasma.

4.1.2 TONSIL COHORTS (I, II)

Two separate tonsil cohorts were analyzed in Studies I and II. For both, tonsillar samples and corresponding sera were obtained from the Department of Otorhinolaryngology-Head and Neck Surgery of Helsinki University Hospital. Patients had undergone tonsillectomy due to chronic tonsillitis or tonsillar hypertrophy. In Study I, altogether 208 subjects with a mean age of 22 years (range; one month to 57 years) were investigated. The tonsils were stored in small pieces at -80°C immediately after surgery and until DNA extraction. In Study II, fresh tonsils from altogether 77 individuals with mean age 22 years (range 2 to 69) were obtained. These samples were processed for virus analysis immediately after surgery.

4.1.3 BONE AND BONE MARROW COHORTS (III, IV, V)

In Study III, 106 bone samples (*femur* (79), *humerus* (10), *tibia* (5), *radius* (4), *fibula* (3), *ulna* (2), *calcaneus* (1), *clavicula* (1) and *os temporale* (1)) from anonymous World War II (WWII) casualties were analyzed. The bones had remained in the battlefields near the border of Finland and Russia until repatriation to Finland at 2007-2012. The remains were initially considered Finnish in origin based on visual inspection of vestiges of military uniforms, ID tags, or personal belongings[197] under the coordination of the Association

for Cherishing the Memory of the Dead of the War. For identification purposes, human DNA profiling was performed at the Department of Forensic Medicine, University of Helsinki, Finland.

In Studies IV and V, femoral bone and bone marrow samples from 27 recently deceased individuals were collected during medico-legal autopsies at the Department of Forensic Medicine, University of Helsinki. The bone marrow was collected from the diaphysis of the femoral bones. The mean age of the cadavers was 68 years (range, 36 to 85), and 19/27 were male and eight female. The mean postmortem interval (PMI) was eight days (range, four to 30). In a single individual, the medical history revealed viral infection (reactivation of VZV on the facial skin).

4.2   CELL LINES (I, II, III)

DNA of A549 cells was utilized as human DNA control throughout the PCR method optimizations (Studies I and III).

Two human B-cell lines, GM12878 and Raji cells (GM04671; both from Coriell Institute), and a monocytic cell line, U937 (ATCC CRL-1593.2) were used in Study II. Cells were cultured in RPMI 1640+GlutaMAX-I medium (Gibco), 1 % penicillin-streptomycin (Sigma-Aldrich), and complement-inactivated 10 % FBS (Gibco) at +37 °C and 5 % CO_2.

4.3   PLASMID CLONES (I-V)

Plasmid clones were used in this thesis work as qPCR templates. The B19V plasmids are presented in detail in Table 4. Other plasmids included pMIXI-III (for HHVs), pMCPyV-LT, and pJCPyV (all from reference[198]), pRNaseP[199], pEBV[200], and pTTV10B cloned from a blood sample (GenBank accession number MT448658).

The copy numbers of plasmids were calculated based on their spectrophotometrically obtained DNA concentrations and weights. Ten-fold dilution series (containing 1E6-1E1 copies / μl) of each plasmid were used as quantification standards in each qPCR. All the plasmid dilutions were stored at -70∘C in 20-100 μl aliquots and thawed only a maximum of three times.

Table 4. B19V plasmid clones used in this thesis work.

Plasmid	Insert	Nucleotides*	Study	Reference
pB19	B19V GT1	180-5416	I-V	Brunstein *et al.* 2000[201]
pLaLi	B19V GT2	105-5147	I	Hokynar *et al.* 2002[28]
pV9	B19V GT3A	282-5314	I	gift from A. Garbarg-Chenon
pD91.1	B19V GT3B	1-5028	I	

* nucleotide numbering according to the reference sequence AY504945.
GT=genotype

4.4 TONSIL CELL SORTING (II)

The two different sorting methods of the primary tonsillar cells are described in detail in Study II Methods at the end of this thesis.

Fresh tonsil pieces were cut into smaller pieces with disposable scalpels, mechanically homogenized with a syringe plunge, treated with 0.1 mg/ml Liberase TL (Roche) at +37 °C for 30 min, washed with PBS, and filtrated through a 70 mm nylon mesh (Corning Life Sciences). The B, T and monocyte/macrophage (M) cell fractions of homogenized and collagen-treated cell suspensions were enriched using anti-CD19, -CD3, -CD14 magnetic beads (Invitrogen), respectively, following manufacturer's protocol. A flow chart of the protocol is shown in Figure 10.
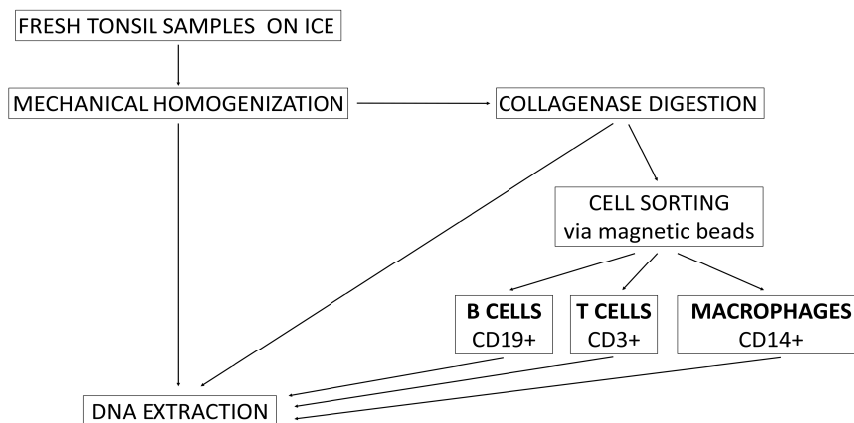

Figure 10. Fresh tonsil cell sorting with magnetic beads.

The subsorting of the B cells of 12 study subjects' collagenase-treated tonsillar cell suspensions in flow cytometer cell sorter was done as follows. The cell suspensions stored in -196 ℃ were first washed with PBS, then stained with 1:100 dilution of anti-CD19-PE (#ab1168, Abcam), -CD27-FITC (#ab30366, Abcam), -IgD-APC (#561303, BD Pharmingen) on ice in the dark for 30 min, washed three times with PBS, and finally sorted in a BD Influx flow cytometer cell sorter (BD Biosciences). The sorting was based on gated CD19+ cells. From the four subpopulations collected in PBS according to the expression of either CD27 or IgD markers (CD27$^+$ IgD$^-$, CD27$^+$ IgD^{low}, CD27$^+$ IgD^{high}, and CD27$^-$ IgD$^+$), total DNA was extracted and analyzed with Pan-B19V-qPCR (see section 4.7.1)

4.5 BONE SAMPLE PREPARATION (III, IV)

For each individual, 5-10 cm piece of bone was obtained for DNA analyses. Bones were cleaned mechanically using a toothbrush and 0.1 % sodium hypochlorite, distilled aqua, and 96.1 w-% ethanol. After the washing steps, bones were let to dry for several days at room temperature under a flow in a laminar hood. Bone powder (0.1-0.5 g) for the DNA extraction was sampled using a dentist drill (W&H Dentalwerk or Schick Qube) or with cryomill (Spex 6775 Freezer/mill; Spex).

4.6 DNA EXTRACTION (I-V)

Table 5 summarizes the DNA extraction methods used in this thesis. DNA was extracted from HUSLAB sera, plasma pools, the WHO Reference Panel, frozen tonsils, and cadaver bone marrows without any pretreatment. Fresh tonsil samples and bones were treated manually before DNA extraction (see sections 4.4 and 4.5). Negative extraction controls (plain buffers) were run in parallel with the study samples.

Table 5. DNA extraction protocols of different sample cohorts.

Cohort	DNA extraction method	Manufacturer	Study
HUSLAB sera, Plasma pools, WHO B19V Panel	DNA Blood Mini Kit	Qiagen	I
Frozen tonsils	MagNA Pure 96 DNA and Viral NA Small Volume Kit	Roche	I
Fresh tonsils	magnetic bead -sorted: KingFisher Cell and Tissue DNA Kit and KingFisher Duo system	Thermo Fisher Scientific	II
	flow cytometry -sorted: DNA mini kit	Qiagen	
WWII bones, Cadaver bones	In-house extraction method combined with QIAquick PCR Purification Kit^	Qiagen	III, IV
Cadaver bone marrows	DNA Mini Kit	Qiagen	V

4.7 QUANTITATIVE PCRS (I-V)

qPCRs for various targets were used throughout this thesis. For B19V, two qPCRs were developed (see section 4.7.1 B19V qPCRs). Furthermore, human RNaseP-qPCR was set up to measure the human single copy RNaseP gene to normalize the viral copies per 1E6 human cells. The qPCRs and corresponding primers and probes are summarized in Table 6.

To avoid PCR contamination, strict precautions were applied throughout the experiments: single-use disposable plastics and filter tips were utilized, and reaction mixes were prepared in a separate clean room, used explicitly for PCR work. The template was added as a final component in a laminar hood dedicated to PCR work, and negative controls were included in all analyses.

Table 6. qPCR primers and probes used in this study.

qPCR	Oligo name	Target sequence (5'-3')	Organism	Study	Reference
Pan-B19V qPCR	B19MT1	CCACTATGAAAACTGGGCAATA	B19V	I-V	this thesis, Study I
	B19MT2	GCTGCTTTCACTGAGTTCTTCA			
	B19MTP1	FAM-AATGCAGATGCCCTCCACCCAG-BHQ1			
VP-qPCR	B19MT9	CATGCCTTATCAYCCAGTARCAGT	B19V	III	this thesis, Study III
	B19MT12	AGGCCCAACATAGTTAGTACCG			
TTV-qPCR	AMTS	GTGCCGNAGGTGAGTTTA	TTV	IV, V	this thesis, Study IV, modified from Maggi et al. 2001[202] and Pistello et al. 2011[203]
	AMTAS	GGACTGGCCGGGCT			
	AMTAS gr4	GGTCTGGCCGGGCT			
	AMTPTU P	FAM-TCAAGGGGCAATTCGGGGCT-BHQ1			
MCPyV LT-qPCR	MCPyV-LT-F	CCACAGCCAGAGCTCTTCCT	MCPyV	IV, V	Goh et al. 2009[204]
	MCPyV-LT-R	TGGTGGTCTCCTCTGCTACTG			
	MCPyV-LT-P	FAM-TCCTTCTCAGCGTCCCAGGCTTCA-TAMRA			
BKPyV-qPCR	V3a-F	CCTTACCCAATTTCCTTTTGCT	BKPyV	IV, V	Hoffman et al. 2008[205]
	V3a-R	ATACATAGGCTGCCCATCCAC			
	V3a-Prb	FAM-TGACCTTATAAACAGGAGAACCC-BHQ1			
JCPyV LT-qPCR	JCPyVLT-F	CTAAACACAGCTTGACTGAGGAATG	JCPyV	IV, V	Dumoulin et al. 2011[206]
	JCPyVLT-F	CATTTAATGAGAAGTGGGGATGAAGAC			
	JCPyVLT-P	FAM-TAGAGTGTTGGGATCCTGTGTTTTCATCATCACT-BHQ1			
EBV-qPCR*	EBV-F	CGGAAGCCCTCTGGACTTC	EBV	II, IV, V	Aalto et al. 2003[200]
	EBV-R	CCCTGTTTATCCGATGGAATG			
	EBV-P	FAM-TGTACACGCACGAGAAATGCGCC-TAMRA			

qPCR	Oligo name	Target sequence (5'-3')	Organism	Study	Reference
	HSV-1FWDLP1	GTTGAGCTAGCCAGCGA	HSV1		
	HSV-1REVLP1	CGTTAAGGACCTTGGTGAGC			
	HSV-1ProbeLP1	FAM-CGCGAACTGACGAGCTTTGTG-BHQ1			
	HSV2 FWD-2-2	CACACCACACGACAACAA			
	HSV-2REVLP1	TAGTTCAAACACGGAAGCC	HSV2		
	HSV-2probeLP1	JOE-CGGCGATGACGGCAATAAA-BHQ1			
	VZVFWDLP1	GCGCAAGGCTATTAGAGC			
	VZVREVLP1	ACATGGCAGAAATCCCTG	HHV3		
	VZVprobeLP1	TxRd-CGCATACCCGGAAGTTCTTCAGAT-BHQ2			
	H5 FWD211	GTGYTCCGTGAATCGTTAC			
	H5 rev 211	AGTCKACCTCGATATCACAAGTCG	HHV5		
	H5 Probe 20	TxRd-ACCCTGCTGCCGCCAGT-BHQ2			
HERQ-9*	HHV6A FWD1-3	CGGCCTCCAGAGTTGTAA		IV, V	Pyöriä *et al.* 2020[198]
	HHV6A REV 10	TGTCCCTTCAACTACTGAATC	HHV6A		
	HHV6A Probe A1^	FAM-AC[+A]T[+G]TTGC[+T]A[+G]AAA[+G][+A]CT-BHQ1			
	HHV6A Probe A2^	FAM-AC[+A]T[+G]TTGC[+T]A[+C]AAA[+G][+A]CT-BHQ1			
	HHV6b FOTY1	TTTGACAGGAGTTGCTGAG			
	H6B ROTY 1	GGATTCAGGAAAAAGGTTCTAA	HHV6B		
	H6B PROBE MVP	JOE-AGGAAGCGTTTCGGTACACTTGGAG-BHQ1			
	HHV7 1. FWD	CTCGCAGATTGCTTGTTG			
	HHV7 1. REV	GCATACACCAACCTACTGTAA	HHV7		
	H7 MOP PROBE	TxRd-TTAGGCATCACGTTGGCATTG-BHQ2			
	HHV8 fwd 3.1	ATATACGGCGACACTGACTC			
	HHV8 REV 10	GAGCAGAAGGCACTTGAAG	HHV8		
	H8 Probe 300	JOE-CGGAGGAGCTAGCGTCAATCA-BHQ1			

qPCR	Oligo name	Target sequence (5'-3')	Organism	Study	Reference
RNaseP-qPCR	RNaseP-FWD	GAGGGAAGCTCATCAGTGGGG	human (RNaseP gene)	I, II, IV, V	This thesis, Study I, modified from McNees et al. 2005[199]
	RNaseP-REV	CTTGGGAAGGTCTGAGACTAGGG			
	RNaseP-P	FAM-AGTGCGTCCTGTCACTCCACTC-BHQ1			

* EBV-qPCR was used as singleplex in Study II and as part of HERQ-9 multiplex qPCR in Studies III and V
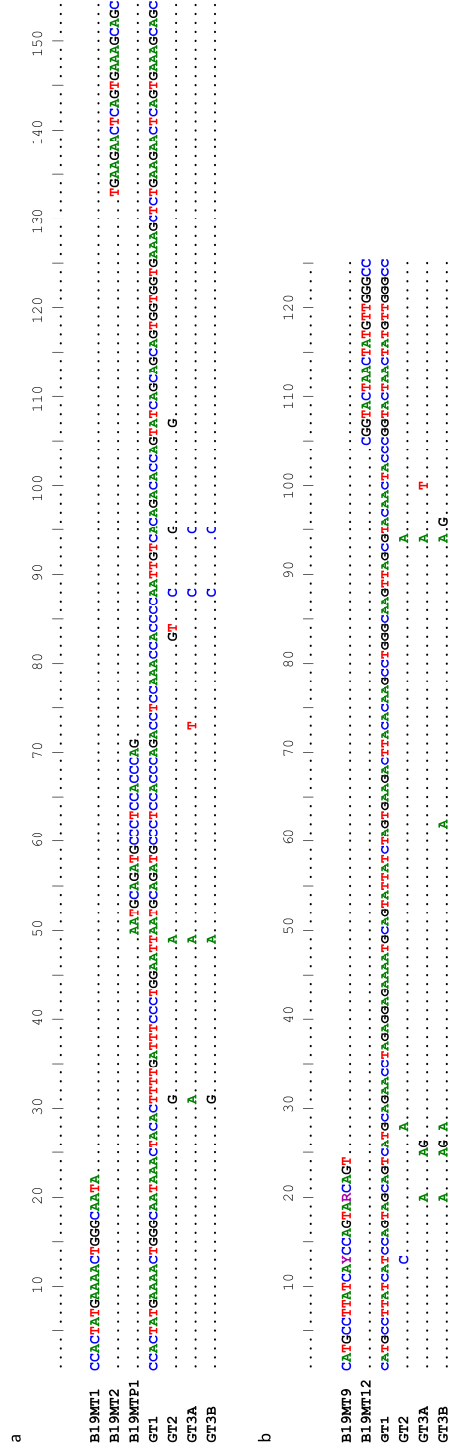
^ Nucleotides in [ ] refer to locked nucleic acids

a

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                 10        20        30        40        50        60        70        80        90       100       110       120       130       140       150
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B19WT1      CCACTATCGAAAACTGGGCAATA.
B19WT2      ...............................
B19WTP1                                          AATGCAGATGCCTCCACCCAG.
GT1         CCACTATGAAAACTGGGCAATAAACTACACTTTTGATTCCCTGGAATTAATGCAGATGCCTCCACCCAGACCTCCAAACCACCCCAATTGTCACAGACACCAGTATCAGCAGCAGTGGTGGTGAAAGCTCGAAGAACTCAGTGAAAGCAGC
GT2         .........G...........A................................GT..C.........G.
GT3A        .........A...........A.......................T.........C.
GT3B        .........G...........A................................C.
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b

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                 10        20        30        40        50        60        70        80        90       100       110       120
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
B19WT9      CATGCCTTATCAYCCAGTARCAGT.
B19WT12     CATGCCTTATCATCCAGTAGCAGTCATGCAGAACCTAGAGGAGAAAATGCAGTATTATCTAGTGAAGACTTACACAAGCCTGGGCAAGTTAGCGTACAACTACCGGGTACTAACTAATGTTGGGCC
GT1         ....C........A..................................................................A.............................CGGTACTAACTATGTTGGGCC
GT2         ...........................................................................A..T...
GT3A        ...........A...AG..................................................A..................A.
GT3B        ...........A...AG.A............................................A.G.
```

Figure 11. Amplicons of Pan-B19V qPCR (a) and VP-qPCR (b). The nucleotide differences of genotypes are shown with sequences AY504945.1, AY044266.2, AJ249437.1 and AY083234.1 for genotypes 1, 2, 3A and 3B, respectively.

4.7.1 B19V QPCRS

Two different qPCR methods for the detection and quantification of B19V genomes were developed in this thesis (Fig. 11).

Pan-B19V qPCR, developed in Study I, is based on the hydrolysis-probe format. The qPCR was designed to target the NS1 region of the B19V genome and to recognize all B19V genotypes. For the primer and probe design, 61 full length or near full-length (coding region) B19V genomes were obtained from the NCBI GenBank nucleotide database, aligned with ClustalW2 (EMBL-EBI) and compared in detail in BioEdit (Ibis Biosciences). Examinations of the secondary structures of oligonucleotides and amplicon-areas were performed with RNA fold WebServer (http://rna.tbi.univie.ac.at). Of the reference genomes, ten represented genotype 2, seven genotype 3A, nine genotype 3B, while the remaining were of genotype 1. Oligonucleotides with no degenerative nucleotides and specific to B19V via BLAST analysis were chosen.

The amplicon area was chosen to distinguish the B19V genotypes by Sanger sequencing of the qPCR product. The nucleotide differences of the genotypes in this region are shown in Figure 11.

After optimization, the Pan-B19V qPCR reaction consisted of 1x Maxima qPCR Master mix (Thermo Scientific) with 0.03 μM of ROX passive reference dye, 0.4 μM each of the forward and reverse primers, 0.15 μM probe, 5 μl template, and nuclease-free water to a final volume of 25 μl. After an initial denaturation at 95∘C for 10 min, the qPCR cycles were 95∘C for 15 s, 60∘C for 15 s and 62∘C for 30 s, altogether 45 cycles.

In Study III, another qPCR, VP-qPCR, was designed to amplify the VP1u region of the B19V genome using the same strains as with Pan-B19V qPCR. The VP-qPCR was designed on SYRB green -format, where the DNA-binding dye gives an increasing light signal if the DNA is amplified. The specific parameters of VP-qPCR are described in Study III Methods et the end of this thesis.

Both of the qPCRs were initially optimized for the Stratagene qPCR thermal cycler (Agilent Technologies) and later, their performance was also evaluated in the AriaMx qPCR system (Agilent Technologies). In both assays, the data analysis was performed with the adaptive fluorescence baseline automatically calculated by the qPCR machine, and the threshold for the quantification cycle (Cq) was set at 10 times standard deviation of mean fluorescence baseline in cycles 5–9.

4.8 SEQUENCING AND PHYLOGENETIC ANALYSIS (I-V)

Both Sanger sequencing (Studies I, II, III) and Illumina-based next-generation sequencing (NGS) (Studies IV and V) techniques were used in this thesis.

In Studies I and II, Sanger sequencing and B19V genotyping were performed on 154 nt region of the NS1 gene, corresponding to the Pan-B19V qPCR amplicon. In Study III, in addition to 154 nt NS1 amplicon, a 121 nt region of VP1u, corresponding to the VP-qPCR amplicon was sequenced. Amplicon areas are presented in Figure 11, on page 43.

In Studies IV and V Illumina-based NGS was performed for simultaneous sequence analysis of altogether 38 viral genomes. To this end, the libraries were prepared with KAPA HyperPlus kit (Roche), and targeted enrichment of viral DNA was performed with custom-designed MyBaits V4 kit (Arbor Biosciences) as described in detail in Study IV Methods at the end of this thesis. The enrichment baits were designed according to the reference sequences for B19V, HBoV1-4, CuV, HPV (types 2, 6, 11, 16, 18, 31, 45), 13 HPyVs, HHV1-8, HBV, TTV, and variola virus (shown in the supplement file of Study IV). The enriched libraries were sequenced on NovaSeq 6000 (SP PE151 reagent kit; Illumina).

From the NGS data of Studies IV and V, the viral genomic sequences were reconstructed using an in-house bioinformatics pipeline TRACESPipe[207] available for download at https://github.com/viromelab/tracespipe. The architecture of TRACESPipe is presented in Figure 12. The consensus, as well as individual sequences (when in low coverage), were confirmed by BLAST (NCBI), and the highest similarity was used to determine the virus genotype. Altogether 17 reconstructed viral sequences are available at NCBI GenBank under accession numbers MT410184-MT410193 and MT988397-MT988403 (submitted, not yet publicly available).

For the phylogenetic analyses performed in Studies I and III, Mega6 package[208] and BEAST package v.1.8.1[209] were utilized. Details for each analysis are given in the original publications at the end of this thesis.
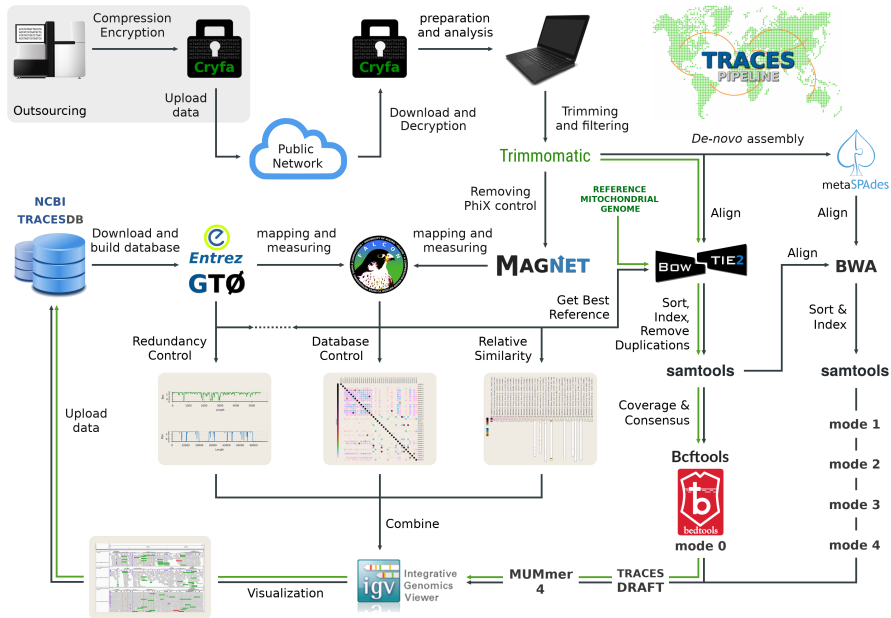
Figure 12. The architecture of TRACESPipe for identification, reconstruction, and analysis of viral and human mitogenomes at multi-organ level. The tools are represented with the respective logos and names. Green arrows indicate mitogenomes, and black arrows indicate viral flowline. Figure reproduced from Pratas *et al.*[207].

4.9 CELL CULTURE ASSAYS (II)

The effect of B19V IgG antibodies on the internalization of B19V-VP2-VLP and live B19V virus was tested on GM12878 and Raji B cell lines as well as on primary tonsillar B cells.

Cell culture methods and controls are described in detail in Study II Methods at the end of this thesis. In brief, in each well 1.50E5-5E6 cells were cultured with 100 ng of VP2-VLP-AF647 or live virus at M.O.I of 10, and incubated with either purified human IgGs (purified IVIG preparation of known B19V reactivity (Gammagard, S/D, Baxter)) or pooled sera from B19V past immunity individuals. Negative controls included purified B19V-negative IgGs and pooled sera from B19V-seronegative individuals. All sera and purified immunoglobulins were complement-inactivated. The uptake was evaluated at 16 h with VLPs or 40h with the live virus. Following incubation, the cells were

treated with 0.25 % trypsin-EDTA (Gibco) to release the cell-bound non-internalized particles.

After trypsin digestion, ADE was determined in the VP2-VLP-AF647 treated cultures by measuring the mean AF647 fluorescence signal with the BD Accuri C6 flow cytometer (BD Biosciences). With live B19V, total DNA from the cells was extracted with the DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions for viral DNA, and the viral copies were quantified by the Pan-B19V qPCR and correlated to 1E6 cells via RNaseP-qPCR.

The Fc-receptor blockage assays were performed similarly, except pre-incubation with anti-CD32 (#551900, BD Biosciences) prior to B19V-VLP or live virus incubation was performed.

4.10 SEROLOGICAL ASSAYS (I, II)

B19V antibody assays (IgG and IgM-EIAs) were performed in Studies I and II, as described in references[210,211]. In brief, for the B19V-IgG EIA, 45 ng per well of biotinylated VP2 VLPs were coated onto streptavidin-coated plates using sample Diluent (#6111040, Anilabsystems). The serum samples were diluted 1:200 and detected with horseradish-peroxidase conjugated anti-human IgG (Dako). For the B19V-IgM EIA, microtiter plate wells (Costar, Corning) were coated with goat anti-human IgM (#55097, Cappel/MP Biomedicals) and blocked with 3 % bovine serum albumin. The serum samples were diluted 1:200 in PBS-Tween, followed by the addition of 10 ng per well of biotinylated VP2 VLP. For detection, a 1:12000 dilution of streptavidin horseradish peroxidase conjugate (Dako) was added. Both EIAs were developed by adding o-phenylenediamine dihydrochloride (Dako) and H_2O_2. The absorbances were read at 492 nm using a Labsystems Multiscan EX (Thermo Fisher). All serum samples were tested in duplicates.

4.11 STATISTICAL ANALYSES (II, IV)

Statistical differences were calculated in Study II with Student's t-test in RStudio (v1.0.153) and with Mann–Whitney U asymptotic sig. (two-sided test) in SPSS (v22.0) and Study V with Student's t-test in RStudio (v3.2.4).

4.12 ETHICAL ASPECTS

All studies were conducted under the relevant guidelines and regulations. The ethics committee of Helsinki and Uusimaa approved the studies (553/E6/2001, 294/13/03/02/2013, 164/13/03/00/2014). The National Supervisory Authority for Welfare and Health (Valvira) approved the research from tissue materials. Written informed consent was obtained from the tonsillectomy patients or in cases of children from their guardian. Virus studies from the WWII casualties were performed as part of the human identification process coordinated by the Association for Cherishing the Memory of the Dead of the War.

5 RESULTS

5.1 PAN-B19V QPCR (I)

In Study I, a qPCR assay for B19V genotypes, called Pan-B19V qPCR, was developed and the performance of the method evaluated with various sample materials. Comprehensive *in silico* -analysis of published B19V sequences was performed to design a primer pair and a probe that amplify and detect all B19V genotypes equally. Furthermore, post-qPCR, the B19V genotypes were distinguished by Sanger sequencing the qPCR-product.

The analytical sensitivity and specificity, as well as the robustness of the method, were determined with B19V genotype plasmids. The analytical sensitivity was ≤10 copies/reaction for all three B19V genotypes. Neither intermixing of the genotypes nor the presence of human DNA interfered with the quantification of B19V DNA.

Equal detection and quantification efficiency for all B19V genotypes was demonstrated with the genotype plasmids pB19, pLaLi, pV9, and pD91.1. Short-term repeatability (intra-assay variation) and long-term reproducibility (inter-assay variation) were determined with pB19 (genotype 1) plasmid, with five replicates and in three separate qPCR runs (Table 7). The method showed high robustness and reproducibility.

Table 7. Short-term repeatability and long-term reproducibility of Pan-B19V qPCR.

Copies / µl	Repeatability		Reproducibility	
	CV (%)	MAD	CV (%)	MAD
pB19 1E1	56.28	0.44E1	42.63	0.33E1
pB19 1E2	3.55	0.03E2	8.16	0.07E2
pB19 1E3	5.99	0.05E3	6.20	0.05E3
pB19 1E4	15.49	0.12E4	18.48	0.13E4
pB19 1E5	11.43	0.09E5	8.23	0.07E5
pB19 1E6	5.43	0.04E6	11.67	0.10E6

CV (%) = the ratio of the standard deviation to the mean

MAD = mean absolute deviation, defined as $\sum |x_i - \bar{x}|)/n$, where x_i is the measured quantity of DNA and $\bar{x}$ is the mean of the n replicates

The quantification accuracy of Pan-B19V qPCR was shown with three B19V-DNA positive (genotype 1, 2, and 3A viruses) plasma samples (Member 1-3) of the WHO International Reference Panel for Parvovirus B19 Genotypes. The results were given in International Units (IU), yielding the correlation factor (0.9 IU/copies). Pan-B19V qPCR showed for the Member 1-3 samples B19V DNA quantities of 1.18E6, 1.83E6, and 1.38E6 IU/ml plasma, respectively. The corresponding mean quantities reported by the manufacturer are 9.55E5 (range 4.07E5-2.09E6), 8.71E5 (2.69E5-3.31E6) and 9.33E5 (1.62E5-3.47E6) IU/ml[196]. The panel samples were correctly identified by Sanger sequencing of the qPCR products as genotypes 1, 2, and 3A (Fig. 13). Member 4 of the panel, a B19V-negative plasma sample, tested negative in Pan-B19V qPCR.
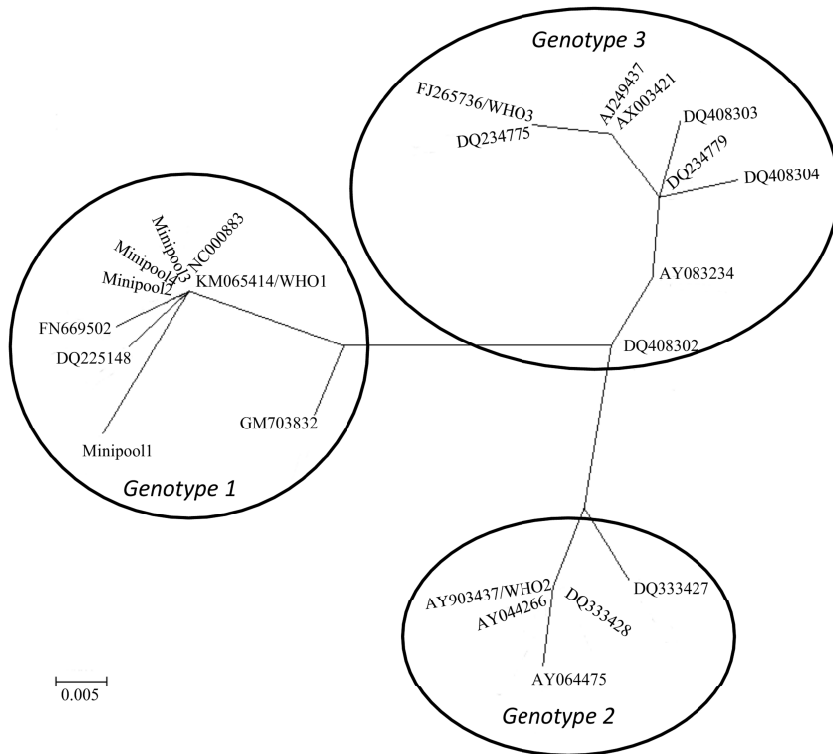
Figure 13. Phylogenetic tree of Pan-B19V qPCR sequences of the WHO Reference Panel (KM065414/WHO1, AY903437/WHO2, FJ265736/WHO3) and the B19V-DNA containing plasma minipools. Figure reproduced from Study I with permission from the copyright holder (Elsevier).

In addition to the WHO Reference Panel, plasma minipools tested for B19V DNA by the Finnish Red Cross upon plasma product manufacturing were investigated with Pan-B19V qPCR. Among 208 minipools four contained B19V DNA according to the Pan-B19V qPCR, with quantities of 5.36E2, 3.09E2, 2.99E2, and 9.27E1 IU/ml pool. Only the first of these had been considered positive at Finnish Red Cross (7.36E02 IU/ml). However, their method reports exclusively DNA levels exceeding cut-off (5.0E2 IU/ml). All four B19V-DNA containing minipools harbored genotype 1 (Fig. 13).

The serum samples analyzed in Study I comprised 20 B19V acute- and 20 B19V past-infection samples. In Pan-B19V qPCR all the former, B19V IgM-positive sera, were B19V-DNA positive with quantities ranging from 3.08E4 to 4.21E8 copies/ml serum. The latter, IgG-positive IgM-negative samples, were all B19V-DNA negative.

The Pan-B19V qPCR was demonstrated to detect low-level B19V DNA also in the presence of human DNA. This was shown with pB19 plasmid spiked with human A549 cell-derived DNA and with *ex-vivo* tonsillar tissue samples. The exogenous human DNA did not influence amplification of the specific product; and the human DNA as template without B19V DNA was not amplified. B19V-DNA prevalence in the tonsillar tissue samples was 35 % (73/208), and all the 73 subjects were B19V-IgG seropositive. On the other hand, all of the 133 B19V-seronegative individuals were qPCR negative in tissue.

5.2   B19V DNA IN HUMAN TONSILS (I, II)

The DNA prevalences and copy numbers of B19V were investigated in two tonsillar tissue cohorts: frozen tonsils (Study I) and fresh tonsils (Study II). All the tissue samples were analyzed with Pan-B19V qPCR as well as human RNaseP gene qPCR to correlate the viral copy numbers to the cell counts.

The B19V-DNA prevalence in frozen tonsils was 35 % (72/208) and in the fresh tonsils, 43 % (33/77). These B19V-DNA tissue prevalences showed 100 % correlation with IgG serostatus.

The median B19V copy numbers in the frozen (n=72) and fresh (n=33) tonsil cohorts were 2.0E0 and 4.5E1 / 1E6 cells, respectively (Table 8). In the frozen tonsil cohort, B19V levels were correlated with age: the highest viral copy numbers occurred in children <10 years of age (n= 5; median 2.9E6 copies / 1E6 cells). In Study II only two children <10 years were included. The two had

at least one log higher B19V DNA loads than did the older subjects. All the children were B19V IgM negative and IgG positive.

Table 8. B19V copy numbers and cell counts in the two tonsil cohorts.

Cohort	n=	B19V DNA copies / 1E6 cells			cells / µl of DNA prep		
		Median	Min	Max	Median	Min	Max
Frozen tonsils (I)	72	2.0E0	1.3E1	3.0E8	3.5E4	7.5E1	1.9E5
Fresh tonsils (II)	33	4.5E1	4.7E-1	3.4E3	2.1E5	1.0E4	5.3E5

5.3   B19V DNA IN TONSILLAR B CELLS AND ADE (II)

The cellular persistence mechanism of B19V in tonsillar tissue was further investigated with the fresh tonsil cohort. We found B19V DNA preferentially distributed in the collagenase-treated B cells (33/77) which contained also the highest viral loads (Fig. 14). In only the two children (<10 years) the highest viral copy numbers were observed in the monocyte fraction.
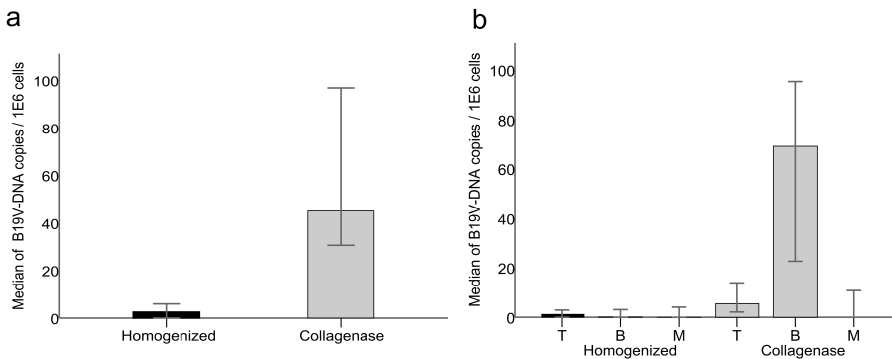
Figure 14. Prevalence and median copy numbers of B19V DNA in (a) homogenized and collagenase-treated cell populations and (b) B and T cells as well as monocyte/macrophages (M). Figure reproduced with modifications from Study II.

The distribution of B19V DNA among B cell subtypes was studied from 12 individuals with the highest B19V copy numbers in the cohort (median 1.06E2 copies/1E6 cells), including the two children <10 years of age mentioned above. In all four sorted subpopulations (1: CD27$^+$ IgD$^-$, 2: CD27$^+$ IgD^{low}, 3: CD27$^+$ IgD^{high}, and 4: CD27$^-$ IgD$^+$), the cell counts were low, and the viral loads fell under the detection limit. The only exception was the two children who had one log higher B19V-DNA copies in the CD27$^+$ IgD^{high} subpopulation compared to other subpopulations (see the supplementary file of Study II at the end of this thesis).

Based on the finding of B19V-DNA persistence in B cells, the virus entry mechanism into these cells was studied with two B cell lines (Raji and GM12878) and also *ex-vivo* isolated primary tonsillar B cells. Virus internalization was followed up using B19V virus-like particles (VP2-VLPs with Alexa Fluor 647) and live (*ex-vivo*) virus from a high-titre viremic plasma. As all tests yielded identical results, for simplicity, only results with Raji cells are presented here. Results with other cells are presented in Study II at the end of this thesis.

Antibody-dependent uptake (ADE) was shown to be a mechanism of B19V entry into B cells with both VP2-VLPs and live virus (Fig. 15a,c). The uptake was blocked with anti-CD32 antibodies (Fig. 15b,d). B19V-capsid internalization was demonstrated by confocal microscopy: the VP2-VLP-AF647 capsids localized within early endosome antigen 1 (EEA1) -labeled endosomes at the cell periphery (Fig. 16).
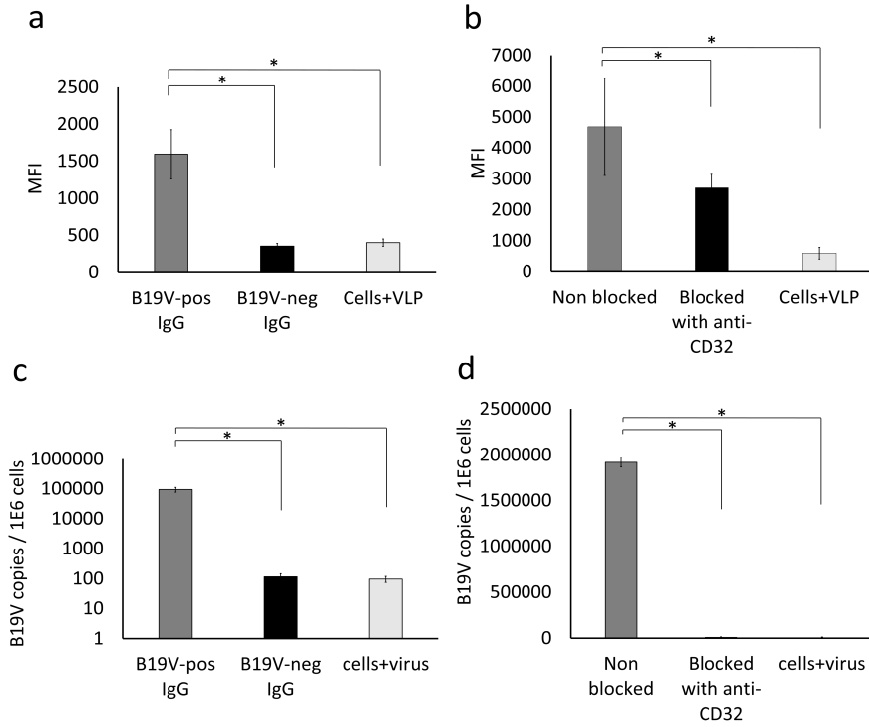
Figure 15. B19V-specific antibodies (1 ug/ml) enhance the uptake of a) B19V VLP and c) live virus into cultured B cells (Raji). The uptake was evaluated at 16 h with VLPs or 40h with the live virus. The uptake is inhibited (b and d) by the addition of anti-CD32 antibodies. * P<0.001. Figure reproduced with modifications from Study II.
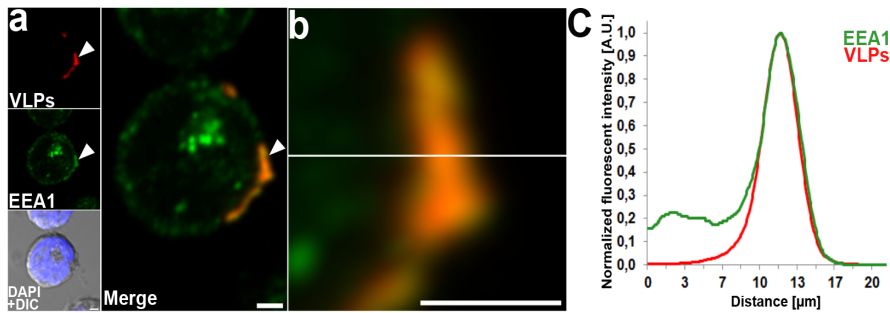
Figure 16. Maximum intensity projections of serial confocal optical sections through (a) Raji cells incubated overnight with VP2-VLP-AF647 (red) and treated with trypsin. White arrows represent enhanced cytoplasmic VLP localization in early endosome antigen 1 (EEA1) positive endosomes (green). Differential interference contrast (DIC) merged with DAPI (blue) images are shown. Area of localization (yellow) (b) and normalized fluorescent intensity profiles in the zoomed area (c) indicate VLP internalization. Scale bars 2 mm. Figure reproduced with modifications from Study II.

5.4 VIRAL DNA IN HUMAN BONE (III, IV)

In Study III, the B19V-DNA prevalence was determined in long bones of 106 anonymous WWII casualties with two qPCRs, Pan-B19V qPCR, and VP-qPCR. In Study IV, from altogether 27 recently deceased individuals, femoral bone specimens were analyzed for 38 persistent viruses with NGS and specific qPCRs, including Pan-B19V qPCR for B19V.

B19V DNA was detected in 45 % (48/106) of WWII casualties (Study III) and 74 % (20/27) of contemporary cadaver bones (Study IV). Among former, we found B19V in the *femur* (34/79 samples), *humerus* (5/10 samples), *tibia* (3/5 samples), *radius* (2/4 samples), *fibula* (1/3 samples), *ulna* (2/2 samples) and *os temporale* (1/1 sample).

In the cadaver cohort, in addition to B19V, altogether 11 different virus species were found in bone (Table 9). The most prevalent virus was TTV (82 %), followed by B19V (74 %), MCPyV (33 %), EBV (26 %), HPV (22 %), HHV7 (19 %), JCPyV (15 %) and HBV (7 %). In addition, isolated examples of HSV1, VZV, CMV, HHV6B, and HBoV1 were encountered. In all, viral DNA was found in 93 % of bone samples. The maximum number of viruses detected in a bone was seven; and on average 2.6 per bone.

Table 9. Viral findings in human bone (Study IV).

Family	Virus	Genoprevalence	Viral load / 1E6 cells
Parvoviridae	B19V	74 %	2.4E3
Herpesviridae	HSV1	4 %	4.9E1
	VZV	4 %	2.0E0
	EBV	26 %	2.1E1
	CMV	4 %	9.8E1
	HHV6B	11 %	-
	HHV7	19 %	-
Polyomaviridae	JCPyV	15 %	2.7E1
	MCPyV	33 %	8.7E3
Papillomaviridae	HPV	22 %	8.7E3*
Hepadnaviridae	HBV	7 %	1.7E3
Anelloviridae	TTV	82 %	1.9E4

* Only HPV type 31 qPCR was performed

B19V viral loads in both bone cohorts were analyzed with Pan-B19V qPCR. RNaseP-qPCR was not performed with the WWII samples, and the viral copies are reported per 1 µg of total DNA. With the cadaver bones, RNaseP-qPCR was done, and the viral copies are reported per 1E6 human cells.

In the WWII cohort, the B19V DNA levels ranged from 3.7E-1 to 4.1E5 copies / 1 µg of total DNA (mean 2.7E4, median 3.0E3). The median B19V copy number in the cadaver bones was 2.4E3 / 1E6 cells. Of other viruses detected in cadaver bone, the median DNA loads for TTV, MCPyV, and EBV were 1.9E4, 8.7E3, and 2.1E1 / 1E6 cells, respectively.

5.5   VIRAL DNA IN HUMAN BONE MARROW (V)

Next, the prevalences of the 38 persistent DNA viruses were investigated in contemporary bone marrow samples. Table 10 describes the viral findings.

Table 10. Viral findings in human bone marrow (Study V).

Family	Virus	Genoprevalence	Viral load / 1E6 cells
Parvoviridae	B19V	63 %	7.9E3
	HBoV1	4 %	-
Herpesviridae	VZV	4 %	2.5E1
	EBV	15 %	9.1E1
	CMV	4 %	1.0E2
	HHV6B	4 %	3.8E1
	HHV7	7 %	3.4E2
Polyomaviridae	MCPyV	11 %	-
Papillomaviridae	HPV31	4 %	-
Hepadnaviridae	HBV	4 %	1.2E3
Anelloviridae	TTV	63 %	2.8E5

In bone marrow, both B19V and TTV DNA-prevalences were 63 %. The two were followed by EBV (15 %), MCPyV (11 %), and HHV7 (7 %). Other viral genomes detected singularly were of VZV, CMV, HHV6B, HBoV1, HPV31, and

HBV. The maximum number of viruses detected in a sample was five, and on average 1.9 per sample.

B19V copy numbers were analyzed and correlated to cell counts as with the tonsillar samples of Studies I and II. The median B19V copies were 7.9E3 / 1E6 cells. The cell count was low in these samples (median 1.3E3 cells / µl of DNA prep). By contrast, the B19V DNA copy numbers were among the highest. Of the other viruses detected, only TTV had higher copies (median 1.4E5 / 1E6 cells). The median copy number of EBV was 9.1E1 copies / 1E6 cells and of HHV7, 1.7E2 copies / 1E6 cells. In the single cases of VZV, CMV, HHV6B, and HBV, the viral loads were 2.5E1, 1.1E2, 3.8E1, and 1.2E3 / 1E6 cells, respectively.

5.6   B19V GENOTYPES IN HUMAN TISSUES (II-V)

B19V genotyping was performed in tissue samples of Studies II-V. In Studies II and III, B19V genotypes were obtained via Sanger sequencing of qPCR products, while in Studies IV and V, Illumina-based NGS was performed, and the genotype was obtained from the samples with >70 % of genome coverage (n=7 (IV) and 7 (V)). Table 11 summarizes the B19V genotype findings of this thesis work.

Table 11. B19V-DNA findings in human tissues organized by genotype (GT) and mean birth year of study subjects.

Cohort	B19V+		GT1+		GT2+		GT3+	
	n=	birth year	n=	birth year	n=	birth year	n=	birth year
Fresh tonsil B cells (II)	33	1983	29	1987	4	1955	0	-
WWII bones (III)	43	1920	0	-	41	1920	2	1920
Cadaver bones (IV)	7	1947	3	1952	4	1944	0	-
Bone marrows (V)	7	1947	2	1951	5	1945	0	-

The genotype distribution according to age groups was determined by comparing the B19V genotype occurrence to the mean birth years of the subjects. In the fresh tonsil cohort (Study II), altogether 33 cases were Sanger sequenced, and the majority were of B19V genotype 1. Four individuals, born in 1947, 1948, 1962, and 1968, carried genotype 2.

Among the WWII casualties of Study III, no genotype 1 was observed. Altogether 41 findings were of genotype 2 and two of genotype 3 (Fig. 17). The year of birth was known with the identified individuals (n=21); and with the unknowns, an estimate was set at 1920. The two genotype 3 sequences were from unidentified individuals.
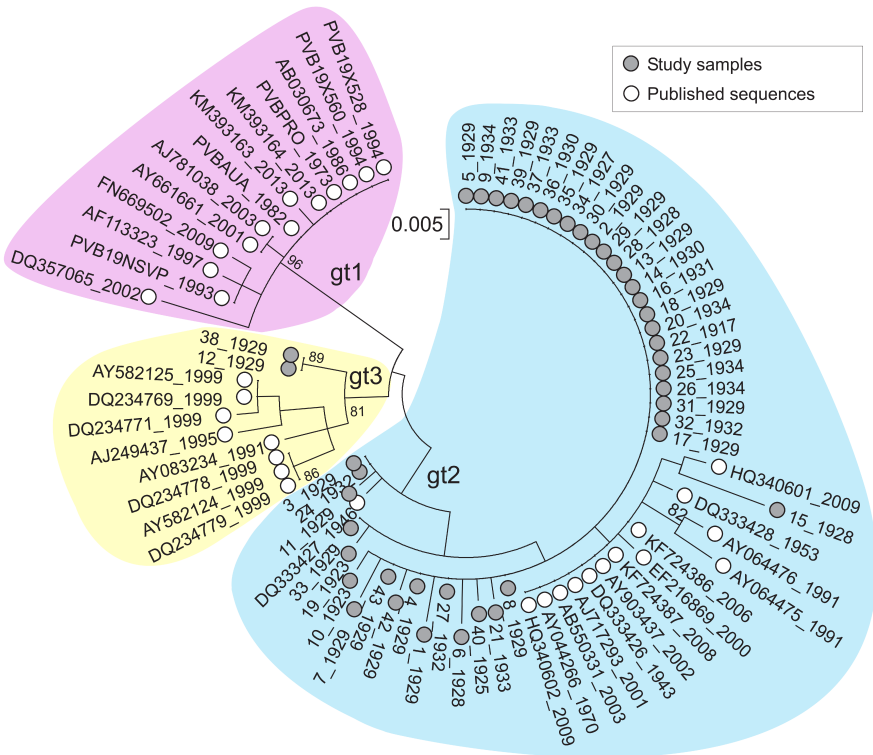
Figure 17. Phylogenetic tree of B19V genotypes obtained from the WWII casualties. Sample dates (plasma representing acute infection) or predicted infection dates (tissue, assumed primary infection at 9 years of age) are shown on labels. Bootstrap resampling values of 70 % or greater are shown. Figure reproduced from Study III.

From the cadaver bone marrows and bones, 14 B19V sequences were constructed: five of genotype 1 and nine of genotype 2. The cadaver subjects (Studies IV and V) were in general older than those of other cohorts, with no difference in genotype distribution. Full or partial B19V ITRs (hairpins) were obtained, including genotype 2 ITR sequences. The sequence analysis revealed that in flip and flop configurations of hairpins, genotype 2 is identical to the genotype 1.

With four cadavers, the B19V genotypes were determined in both bone and bone marrow. The reconstructed B19V sequences within an individual were i) of the same genotype and ii) identical or nearly identical in genomic areas spanned by the two sequences. The numbers of single nucleotide polymorphisms (SNPs) in the sequences in a given individual ranged from one to four.

When combining all the B19V positive individuals included in this thesis, genotype 1 was found among individuals born between 1938 and 2009 (average 1981). All the findings of genotype 2 were in individuals born before 1969 (average 1926, min 1908).

5.7 B19V EVOLUTIONARY ANALYSIS (III)

In the WWII casualties, the overall substitution rate for B19V was estimated using BEAST under several substitution rates and clock models. All models gave similar results (Fig. 18). The best fit model estimate for B19V evolutionary rate was 2.11E-4 subs/site/year (95 % high probability density interval (HPD): 1.18E-4 to 3.16E-4). The most recent common ancestor (MRCA) of all three genotypes was estimated at 1833 (HPD: 1751-1893). Moreover, the MRCA of genotype 2 and genotype 3 were estimated separately at 1868 (HPD: 1823–1902) and 1917 (HPD: 1893–1929), respectively.

a
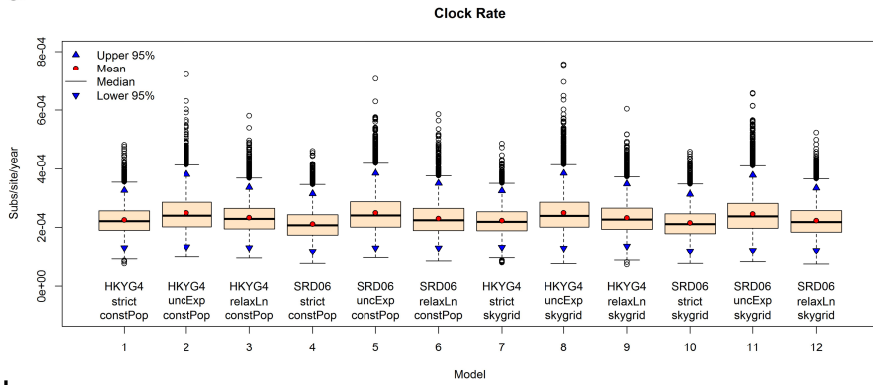
b
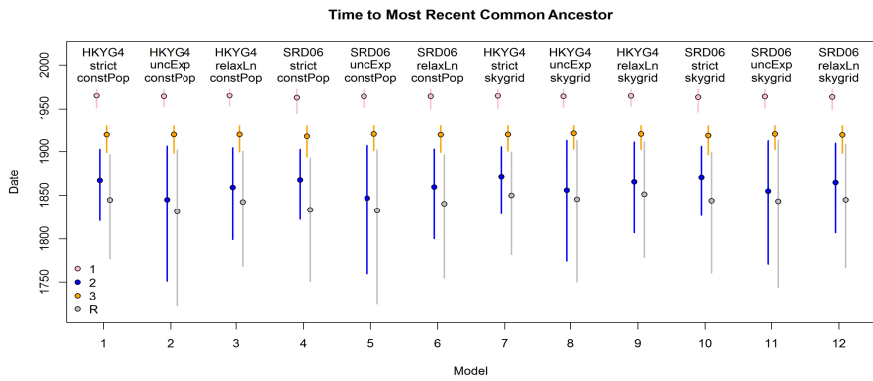
Figure 18. Overall clock rate estimates (a) and time to most recent common ancestors (b) for B19V genotypes 1, 2, and 3 using several models with BEAST. Figure reproduced with modifications from Study III and its supplementary material.

6 DISCUSSION

6.1 QUANTITATIVE PCR FOR B19V GENOTYPES (I)

Human parvovirus B19 (B19V) infection can be transmitted from individual to individual via three routes: respiratory droplets, vertical transmission from mother to fetus, and blood products[62–67]. The viral load in primary infection can be extremely high. B19V DNA is detectable after primary infection in i) blood, for up to weeks or months (sometimes longer), and ii) tissues for decades[43,69–71]. Thus, for B19V diagnosis and research, nucleic acid testing is required, especially quantitative PCRs (qPCR).

In Study I, a new qPCR assay, called Pan-B19V qPCR, was developed. At the beginning of this thesis work, only a few B19V qPCRs existed[36,48,57,211–214]. Moreover, their utility in analysis of tissues or ability to distinguish the B19V genotypes had not been demonstrated. Thus, a new qPCR method suitable for detection and identification of B19V genotypes in clinical materials such as body liquids (plasma, serum) and solid tissues appeared warranted.

The hydrolysis probe-based Pan-B19V qPCR targeting the NS1 area of the B19V genome detected and quantified equally all virus genotypes. The new method was shown to be highly sensitive and specific. The quantification and correct genotyping were validated with the WHO Reference Panel for B19V genotypes. Moreover, the method was tested with a large set of samples, including acute and past B19V-infection serum samples, B19V-seronegative individuals' sera, blood donor plasma pools, and tonsillar tissue samples.

The Pan-B19V qPCR results of the acute- and past-infection serum samples were perfectly in line with the serological results. Traditional diagnosis of B19V infection relies on antibody detection; however, knowledge of the viral DNA load in blood may assist in the timing of B19V infection, especially in immunosuppressed individuals and pregnant women[48,211,215]. In the former, the B19V antibodies may not be produced, or their development may be delayed, leaving qPCR as the only diagnostic tool. In the latter, vertical transmission can lead to fetal loss, especially with maternal infection within the first and early second trimester (<20 weeks[83]), thus calling for careful evaluation of the infection time. However, if the diagnosis of recent B19V infection is based only on the presence of B19V DNA without knowledge of viral load or serostatus, the result may be misleading due to persistence of B19V DNA in blood for months after primary infection (Fig. 3 on p. 21). Therefore qPCR, together with serology, is increasingly recommended for B19V diagnosis[48,211,215].

B19V DNA has been found as a contaminant in blood-derived coagulation factor concentrates, even after the introduction of viral inactivation procedures[47,84,87], and patients with hematological disorders have obtained B19V infection via these products[85,216,217]. Therefore, the Food and Drug Administration of the United States (FDA) and the European Directorate for the Quality of Medicines and Healthcare (EDQM, Council of Europe) introduced B19V nucleic acid testing and quantification as a new step in blood product manufacturing[88]. The utility of Pan-B19V qPCR in plasma monitoring was in the present work shown with donor plasma minipools consisting of 3 328 individual blood donations. Interestingly, all the B19V-DNA positive minipools harbored genotype 1, the currently circulating B19V type. I.e., no genotypes 2 and 3 were found, supporting their extreme rarity in Northern Europe[27,43,218].

The quantitative PCR for B19V genotypes developed in this thesis is suitable for B19V diagnostics and research both in human body fluids and solid tissue samples. Moreover, the method is applicable to monitoring B19V levels of plasma pools during blood product manufacturing. The B19V genotype present in a sample can be identified by Sanger sequencing of the amplified products.

6.2   B19V DNA IN HUMAN SOFT TISSUES AND BONES (I-V)

After primary infection, B19V establishes lifelong genome persistence in various human tissues. This ubiquitous persistence of B19V-DNA has been of wide recent interest, with ~50 % prevalence among grown-ups (Fig. 5 on p. 24). However, its cellular and molecular mechanisms have been unknown.

For B19V-DNA long-term persistence, clinical associations have been proposed, especially with synovia and heart. Over 100 studies or case reports have been published on the role of B19V persistence in heart and autoimmune thyroid diseases, especially dilated cardiomyopathy, yet without proof of causality[219–222]. Quantification of the B19V-DNA levels has been suggested to add information on the clinical impact[148,149,223]. However, in most studies, the DNA levels have not been assessed. In this thesis work, we determined from healthy individuals the B19V prevalences and copy numbers in three organs: tonsil, bone marrow, and bone.

Most B19V persistence studies have been performed with a single biopsy sample per individual. Therefore, more research is warranted to understand

the actual B19V DNA prevalences and levels in the different tissues. So far, only Manning *et al.*[44] have studied B19V DNA tissue distributions within individuals. Among bone marrow, lymphoid tissue, and brain, they found the highest B19V load in the first one. In this thesis, we investigated bone marrows and bones of 27 study subjects. Viral prevalences were moderately higher in bone, e.g., for B19V 74 % vs. 63 % in the bone marrow, while the copy numbers were higher in the bone marrow. To gain a full picture of B19V tissue distribution, one would need to study several organs per individual with both sequencing techniques and qPCR.

6.2.1 B19V PERSISTENCE IN TONSIL AND TONSILLAR B CELLS (I-II)

Table 12 illustrates the literature existing on B19V in tonsillar tissue. Our results in Studies I and II were highly comparable to those in Kakkola *et al.*[126], Norja *et al.*[43], and Şahiner *et al.*[150]. Only Silvoniemi *et al.*[224] detected remarkably lower prevalence (total of 7.2 %); however, they suggested this to be due to methodology and the very low copies. Şahiner *et al.* saw in children lower B19V-DNA prevalences than in adults. This is expected, as also anti-B19V IgG prevalence increases with age. Of children under five years, approximately 2-20 % have B19V-IgG antibodies, while in adolescents and young adults, the B19V seroprevalence is ~40-60 %[77–79].

In addition to us, only Şahiner *et al.*[150] disclosed B19V DNA levels in tonsils, yet without correlation to cell counts. Nonetheless, both they and we report among children than adults significantly higher B19V-DNA levels, as opposed to prevalence. In our Studies I and II, all the B19V-DNA positive children showed past immunity with B19V-IgG positivity and IgM negativity. Thus, the findings do represent B19V persistence and suggest that the copy numbers in tonsillar tissue wane with age, with the high levels detected only shortly (months to a few years) after primary infection.

Indeed, in our tonsil cohorts, we determined the serostatus of all the study subjects. In both cohorts, all individuals who were B19V-DNA positive in tissue were B19V-IgG seropositive. On the other hand, all of the B19V-seronegative individuals were qPCR negative in tissue. Thus, individuals with persistent B19V in tonsils have had primary infection, produced virus-specific antibodies, and cleared the virus from blood (Fig. 3 on p. 21). Nonetheless, the virus is able to establish tissue persistence.

Table 12. B19V-DNA findings in human tonsillar tissue.

Study	Mean age (years)	Healthy / Diseased	Cases	B19V DNA	
				Prevalence	Copies
I	22	tonsillitis/tonsillar hypertrophy	72/208	35 %	2.0E0 / 1E6 cells
II	22	tonsillitis/tonsillar hypertrophy	33/77	43 %	4.5E1 / 1E6 cells
Kakkola *et al.* 2004[126]	19	tonsillitis/tonsillar hypertrophy	3/10	30 %	-
Norja *et al.* 2006[43]	23	tonsillitis/tonsillar hypertrophy	39/220	18 %	-
Şahiner *et al.* 2014[150]	group I: 6	tonsillitis/tonsillar hypertrophy	6/44	14 %	>5E5 copies/ml
	group II: 24		6/12	50 %	<5E5 copies/ml
Silvoniemi *et al.* 2020[224]	group I: <16	tonsillitis/tonsillar hypertrophy	3/37	8 %	-
	group II: 16-30		9/93	9 %	-
	group III: >30		1/51	2 %	-

As B19V spreads via the respiratory route[91], persistence in tonsils, a lymphoid organ that collects pathogens via immune cells[225], seems natural. Yet, a more intriguing possibility would be that the virus enters tonsils because of the long-lived cells[226] permitting persistence for decades. Indeed, the cell type accounting for the lifelong B19V-DNA tissue persistence has not been known before the present work. We hypothesized that long-lived leukocytes might act as a suitable host for the lifelong persistence. In Study II, we found that the B19V DNA occurred preferentially among, and in significantly higher loads within, the tonsillar B cells (CD19+) that could be released by collagenase, pointing to persistence in long-lived B cells, as suggested by Medina *et al.*[226]. Unfortunately, we could not detect B19V DNA in B cell subpopulations sorted based on their expression of CD27 (memory) or IgD (naïve) surface markers. This was probably due to low overall B19V copies: even though we investigated the 12 individuals with the highest copy numbers, the median in the unsorted B cell population was only 1.06E2 copies / 1E6 cells, and division of these into further subpopulations diluted the viral genomes to undetectable levels. Only two children showed detectable viral loads: one log higher viral copies were detected in the CD27+ IgD^high -cells compared to other B cell subpopulations.

These children were B19V-IgM negative and non-viremic, suggesting non-acute albeit relatively recent primary infection.

However, we did encounter B19V genotype 2 – known to have disappeared from circulation before the 1970s[43] – in tonsillar B cells providing further evidence of the persistence occurring in long-lived cells. Even though a few sporadic instances of genotype 2 viremia have been reported (Table 1 on p. 18), their rarity rules against reinfection. Also according to sequence data, B19V does not evolve or reactivate within human tissues[227], and little evidence exists of B19V integration into the host genome[228]. Hence, the four genotype 2 positive subjects, all >45 years of age (born 1947, 1948, 1962, and 1968), must have carried the genotype 2 virus in their B cells for numerous decades, which suggests for these cells a survival time of 40-60 years. Thus, immunological memory is not only maintained in the absence of antigen re-exposure, but may also be due to decades-long survival of the original clones.

6.2.2   B19V PERSISTENCE IN BONE MARROW (V)

Table 13 illustrates the literature existing on B19V in bone marrow. Our B19V-DNA prevalence in cadaver bone marrow (63 %) was among the highest reported. The differences in prevalence might be study group (age or clinical, or small N), sampling or methodology-related. The mean age of Study V was high, 68 years, anticipating high B19V-DNA prevalence; however, we included only 27 individuals.

Altogether four other studies have investigated post-mortem bone marrow samples. Cassinotti *et al.*[229] showed a 9 % B19V-DNA prevalence in victims of traffic accidents. One would assume that they were young because of the low prevalence. Manning *et al.*[44] divided their cadaveric samples into two groups: HIV infected (mean age 54) and HIV uninfected (mean age 34) with B19V-DNA prevalences of 52 % and 75 %, respectively. Eis-Hübinger *et al.*[115] investigated cadavers (median age 62) without signs of acute B19V infection and found 13 % prevalence.

The remaining studies investigated bone marrow aspirates, with B19V prevalences of 2 to 26 %. However, bone marrow aspirates represent mostly red marrow, while samples of autopsy might comprise red or yellow marrow, depending on location. The marrow within long bones is mostly yellow, albeit some red marrow might be present in the middle parts of long bones in young individuals[230]. Moreover, the nature and compartmentalization of these two bone marrow types remain to be fully understood[231,232].

Importantly, most of the prior bone marrow studies were performed at a time when B19V genotype 2 was not known, and the primer pairs mismatched this genotype, which may explain the lower prevalences in older studies[28]. Only the most recent, i.e., by Manning *et al.* in 2007[44], Corcioli *et al.* in 2008[38], and Garcia *et al.* in 2011[121], were conducted with primers amplifying all B19V genotypes. In the three, the DNA prevalences were 13-75 %. The methods used in our study detect all B19V genotypes equally. Indeed, our bone marrow samples in which the B19V genotype was determined, 5/7 harbored genotype 2. The nucleotide composition analysis of these B19V sequences supports that they are unique findings of B19V and not the result of contamination. The persistent B19V DNA seems to be more abundant in bone marrow than previously considered.

During primary infection, human bone marrow is the replication site of B19V[91,92]. As B cells originate from the bone marrow, one might suggest the cellular persistence mechanism to be the same as in tonsils (Study II). Indeed, whether or not B cells account for the B19V persistence in bone marrow deserves investigation. One potential could be the protocol we used for tonsils in Study II: fresh bone marrow aspirates sorted into cell populations immediately after sampling, followed by qPCR, or even single-cell PCR.

However, bone marrow harbors multiple other long-lived cells, such as stem cells and immunological memory cells of many types[233], to act as potential hosts. B19V has been shown *in vitro* to integrate into the host cells of primary infection, EPCs, which might explain the lifelong persistence in bone marrow[228]. Moreover, B19V-DNA has been shown to persist in mesenchymal stromal cells[234,235] (MCS), multipotent, non-hematopoietic cells that reside in bone marrow, differentiate into cartilage and bone[236], and are used as prophylaxis or treatment of graft-versus-host disease[237].

Table 13. B19V-DNA findings in human bone marrow.

Study	Mean age (years)	Study subjects	Sampling method	Cases	Prevalence
V	68	cadavers, no BM related diseases	sampling from within femoral bone in autopsy	13/27	63 %
Cassinotti et al. 1997[229]	group I: unknown	tendon or bone tissue donors deceased in traffic accidents, healthy	sampling from within tendon or bone in autopsy	4/45	9 %
	group II: unknown	patients with clinical suspicion of B19V infection	BM aspirate	17/84	20 %
Eis-Hübinger et al. 2001[115]	62*	randomly selected cadavers, no signs of B19V acute infection	sampling in autopsy	3/23	13 %
Heegaard et al. 2002[238]	28	healthy BM donors	BM aspirate	4/153	2 %
Lundqvist et al. 2005[120]	54	patients with rheumatic disease	BM aspirate	13/50	26 %
Manning et al. 2007[44]	group I: 54	HIV infected	sampling in autopsy	12/23	52 %
	group II: 34	HIV uninfected	sampling in autopsy	6/8	75 %
Corcioli et al. 2008[38]	60	patients with B19V-unrelated hematological disorders	BM aspirate	9/44	20 %
Garcia et al. 2011[121]	group I: 55*	patients with idiopathic cytopenia	BM aspirate	18/120	15 %
	group II: 35*	healthy bone marrow donors	BM aspirate	6/45	13 %
	group III: 60*	patients with oncohematological disorders	BM aspirate	15/120	13 %

* median age

BM bone marrow, HIV human immunodeficiency virus

6.2.3 B19V PERSISTENCE IN BONE AND THE EMERGENCE OF ARCHEOVIROLOGY (III-IV)

B19V persistence in human bone was investigated in Studies III and IV. Even though the B19V DNA has been detected by us and others in a range of tissues and organs, no direct evidence of its persistence in bone existed before our Study III. We found a remarkable prevalence of 45 % in WWII casualties. Considering the decades-long exposure to harsh environmental conditions in the boreal wilderness, this prevalence is astonishingly high. The detection frequency is similar to or even higher than in modern soft-tissue biopsies (Fig. 5 on p. 24). Altogether, we showed that skeletal remains are suitable for investigation of epidemiology and evolutionary history of DNA viruses. Thus, our findings laid the foundation for a new research approach: archeovirology.

Indeed, B19V is the first virus ever to have been found in human bone; and the discovery was followed by hepatitis B virus (HBV)[239]. Immediately thereafter, a paper in Science[240] revealed variola virus (VARV) in two Viking Age skulls. A recent editorial[241] written only a few weeks before our Study IV was published, hypothesizes that viruses may be extremely prevalent in human bone and have a role in the formation of bone diseases. In Study IV we showed, beyond the B19V and HBV, ten different viruses in specimens of contemporary femoral bone. Thus, bone is a much richer source of persistent human DNA viruses than ever perceived. We found that in prevalence B19V, next to TTV, was the foremost, confirming the data of Study III and indicating bone to be another organ harboring B19V.

The structure of bone, with hydroxyapatite stabilizing nucleic acids[242], might help viral DNA to resist decay. Moreover, most of the viruses we detected in Study IV (except HHV6B) characteristically persist in our organs in episomal form. E.g., herpesviruses, polyomaviruses, and papillomaviruses establish latency in densely packed nucleosomes[169,170,243,244]. The circular form with histone packaging may confer to DNA viruses superior preservation, compared to the nuclear and mitochondrial DNAs of the host[245–247].

Human DNA is currently considered best-preserved in *petrous* bone[248] as this particular bone lies deep inside the ear, and thus, is protected from environmental contaminations. So far, only the very recent Viking Age VARV study by Mühlemann *et al.*[240] showed the presence of viral genomes within the *petrous* bone. In Study III while investigating mostly femoral samples, we did find B19V DNA also in *humerus, tibia, radius, fibula, ulna, clavicula,* and *os temporale.* Moreover, both B19V and HBV DNA have been recently detected in dental human remains[41,239]. Thus, further study is warranted to determine

whether viral genomes are ubiquitously spread across human hard tissues and different bone types.

6.3 ANTIBODY-DEPENDENT ENHANCEMENT OF B19V INFECTION (II)

While viruses enter their host cells mainly via specific receptors, for some viruses alternative entry routes do exist. An example is the antibody-dependent enhancement (ADE) of dengue virus. As infection with one dengue virus type produces neutralizing antibodies towards that type, those antibodies do not fully protect against other virus serotypes. Instead, an infection with another virus type can cause more severe disease due to enhanced cellular uptake via cross-reactive non-neutralizing antibodies[249,250].

Because of our discovery of B19V persistence in lymphoid B cells, we investigated the B19V entry mechanism into these cells. We found enhanced virus uptake via the Fc-receptor (CD32) and suggested that B19V is able to enter the B cells via an endocytic pathway.

Some symptoms of B19V primary infection, e.g., arthralgia, might be due to enhanced virus uptake into B cells. B19V primary infection-related arthropathies are allegedly due to IgG deposition[251], and the role of B19V in triggering or exacerbating autoimmune diseases has been proposed[252]. Thus, the finding of B19V ADE in B cells might provide a basis for the prolonged B-cell activation and the sustained polyclonal antibody production characteristic of B19V infection. Even without evidence of causality, understanding of the mechanisms by which B19V may modulate B cells and have a role in development of proliferative disorders calls for further investigation.

B19V genotypes do form a single serotype, and antibodies against one protect against all[35,36]. Therefore, B19V ADE may not facilitate disease. On the other hand, ADE might assist B19V to establish persistence. The long-lasting B19V viremia coincides partially with the antiviral IgG production (Fig. 3 on p. 21). Soon after contagion, the patient has in blood both the virus and low-affinity antibodies. Resilient viremia occurs commonly among viruses utilizing ADE[253], and low-affinity antibodies have been shown to augment pathology, e.g., with human respiratory syncytial virus[110]. Over time, the B19V IgG antibodies mature, and high-affinity neutralizing antibodies prevail, whereby the B19V ADE might happen only shortly after primary infection.

After proliferation in the erythroid progenitor cells of human bone marrow, B19V might enter via ADE some non-permissive cell types, such as B cells, monocytes[106], and endothelial cells[107,223] which could then act as a reservoir of persistence. Further studies on the B19V cellular persistence mechanism in tissues of other types are needed, and the specific antibodies involved in the phenomenon must be identified to understand the significance of ADE in B19V infection.

6.4 B19V MOLECULAR EPIDEMIOLOGY AND EVOLUTION (II-V)

Investigation of epidemiology of pathogens at molecular level contributes to understanding their distribution, circulation, transmission, and causation of disease[254]. As the sequence diversity of viruses derives from mutations accumulated over time, evolutionary analysis of viruses provides information on their past and, thus, may help in assessment of future evolutionary events.

In this thesis work, B19V genotypes present in diverse tissue types and from early 1900s to contemporary times were investigated. The bone marrow and bone samples in Studies IV and V originated from the same cadavers, allowing us to compare the persistent B19V genomic sequences. We observed that within an individual, a single B19V genotype is present. The reconstructed sequences in bone marrow and bone were very similar or even identical: the maximum number of single nucleotide polymorphisms (SNPs) in a given individual's two tissues was four. However, in-depth sequencing studies are needed to understand if B19V quasispecies persist in various tissues of an individual.

Furthermore, we investigated the nucleotide compositions of the ITR-regions (hairpins) of genotype 2. The ITRs are needed in viral replication[255] and therefore are crucial for the virus's survival. Until our Study IV, only a single full-length genotype 2 had been published[40] (GenBank accession number AB550331). In this thesis work, altogether nine full or near-full length genotype 2 genomes were sequenced. The ITR compositions of our genotype 2 sequences were similar to the previously published sequences representing genotypes 1 and 2. Therefore, the ITRs do not appear to play a role in B19V genotype switch. However, until ITR analysis of genotype 3, no definitive conclusion can be drawn. Indeed, a preprint recently appeared in bioRxiv presenting full-length sequencing of ancient genotype 3 strains[41]; however, the sequences are not yet available in Genbank.

B19V genotype 2 has been primarily found in tissues of individuals born before the 1970s[43–45]. In this thesis, B19V genotype 2 was detected only in tissue samples of individuals born before 1969. This included tonsillar B cells of the four individuals born in 1947, 1948, 1962, and 1968. In the WWII cohort of Study III, we exclusively detected genotypes 2 and 3; not a single case of genotype 1 was found. This data supported the hypothesis by Norja *et al.*[43,227] that genotype 1, the currently prevailing form in circulation through the world, emerged after the second World War. The data, for the first time, shed light on the epidemiology of B19V in Northern Europe between ~1923 and 1944.

Soon after our Study III, Mühlemann *et al.*[12] investigated B19V evolution by analyzing ancient human remains. They found ten B19V genomes, of which five were genotype 1 and five genotype 2. All these sequences fell, in the phylogenetic tree, basal to contemporary B19V sequences. Their samples came from Europe, Central Asia, and Greenland. Within Europe, the samples originated from areas of current Russia, the Czech Republic, Poland, the UK, and Sweden. All sequences of genotype 1 were from Central Asia while those of genotype 2 from Europe and Greenland. Geographically closest to Finland was the Swedish sample, originating from the island of Gotland around 1000 years ago and harboring genotype 2. As we did not detect any genotype 1 sequences in Finland in the early 1900s, it is possible that this genotype circulated in some parts of the world (for example, Asia) already much earlier but spread to Northern Europe – and Finland – more recently. Interestingly, Bowen *et al.*[256] found in 2019 that HSV-1 strains circulating in Finland separate into two distinct phylogenetic groups, potentially reflecting historical waves of human (and virus) migration into Finland. Indeed, the Finnish human gene pool has been first influenced by Asian/Siberian Mesolithic hunter-gatherers and later medieval Swedes and Germans[257,258]. This can still be seen as a rather strict geographical East-West division of the Finnish gene diversity[257,259].

The most recent common ancestor (MRCA) of B19V genotypes has been estimated to date back to the 1950s (Norja *et al.*[227]), 1833 (by us, Study III), and 12600 years ago (Mühlemann *et al.*[12]). Likewise, the substitution rates (Table 14) derived from contemporary sequences alone (Shackelton & Holmes[260], Parsyan *et al.*[29] and Norja *et al.*[227]) are higher than those derived from ancient sequences by Mühlemann *et al.*[12]. In our Study III, based on conserved partial sequences of NS1 and VP1 genes, the B19V evolution rate fell closer to the estimate calculated with the contemporary data. Access to ancient viral sequences from individuals having lived hundreds or thousands of years ago can significantly improve the understanding of the timescales of virus evolution. As regards B19V evolution rate and MRCA, more sequences covering the past 13000 years (and beyond) are required. Mühlemann's[12] 10 ancient sequences dated back 500 to 6 900 years. Already their inclusion to

the analysis provided remarkable new information on B19V evolution and revealed that genotype 1 existed far earlier than thought.

Table 14. B19V evolution rates analyzed by the Bayesian Markov chain Monte Carlo (MCMC) method.

Study	Sample material	Genomic region	Nucleotide substitution rate*	
			mean	HPD
III	WWII bones	combined partial NS1/VP1	2.1E-4	1.2E-4 to 3.2E-4
Shackelton & Holmes 2006[260]	contemporary plasma and tissue	VP1	1.1E-4	1.2E-5 to 2.4E-4
		coding region	1.8E-4	9.0E-5 to 2.7E-4
Parsyan *et al.* 2007[29]^	contemporary plasma	NS1	1.2E-4	2.3E-6 to 2.9E-4
		VP1	2.3E-4	2.0E-6 to 5.5E-4
		combined NS1 and VP1	2.0E-4	2.0E-6 to 5.1E-4
Norja *et al.* 2008[227]	contemporary plasma	combined partial VP1/VP2	3.6E-4	2.5E-4 to 4.8E-4
	contemporary tissue		4.4E-5	1.4E-6 to 9.7E-5
	plasma and tissue combined		1.7E-4	1.2E-4 to 2.2E-4
Mühlemann *et al.* 2018[12]	ancient bones	coding region	1.2E-5	1.0E-5 to 1.4E-5

* substitutions/site/year

^ only genotype 3 sequences included

HPD = 95 % high probability density interval

VP1=viral protein 1, NS1=non-structural protein 1

Not a single genotype 3 was encountered in Mühlemann's ancient samples. On the other hand, in 2007, Parsyan *et al.*[29] suggested genotype 3 to be more ancient than the other two, due to the significantly higher diversity within the genotype 3 group. We detected two cases of genotype 3 among the WWII casualties (Study III). This genotype has never before been recorded in Northern Europe but has in Ghana, Brazil, and India (Table 2 on p. 19-20). Thus, the B19V genotype 3 finding provided new insights into the origins of these two unidentified individuals. Consequently, we studied their mitochondrial DNA and Y chromosome profiles and indeed, the data excluded Finnish origin. Those remains likely belonged to soldiers of the Soviet Red Army, comprising individuals of European as well as of Asian origin.

7 CONCLUDING REMARKS AND FUTURE PROSPECTS

In this thesis, parvovirus B19 (B19V) persistence and evolution were investigated. The new qPCR for B19V genotypes developed in this thesis is suitable for research, diagnostics, and monitoring viral levels of plasma pools in blood product manufacturing. Furthermore, we developed a novel NAT method that couples custom viral targeted enrichment to NGS and can simultaneously detect altogether 38 viruses in a sample. An NGS-based assay, which can be used to analyze the complete viral content of a sample, could one day become standard practice in diagnostic laboratories.

The significance of persisting DNA viruses in human organs remains to be fully understood. Especially in immunocompromised patients and in organ transplant recipients, the tissue virome can cause severe complications or even fatal illnesses. Can these viruses be beneficial to us, such as the bacteriome we harbor[261,262]? Or are they mere bystanders, only nucleic acids stored in our organs?

The results of this thesis work open intriguing research avenues and yield new questions of viral persistence. We showed that B19V can persist for several decades in tonsillar B cells and that the viral entry mechanism into these cells is antibody-mediated. Whether or not B cells explain the ubiquitous B19V persistence of all human tissues is an intriguing question. The cell types harboring B19V in other organs need to be identified, and the viral entry mechanisms into these cells elucidated. Moreover, the molecular mechanisms of the B19V persistence are yet to be determined. Our data showed that it is the whole genome of B19V that persists in bone marrow and bone. With modern sequencing techniques, possible integration sites or episomal structures can be revealed.

In addition to persistence, B19V evolution and molecular epidemiology were investigated in this thesis. The B19V types circulating in Northern Europe in the early 1900s were revealed, and the molecular epidemiology of the virus was illustrated. Indeed, we sequenced full-length B19V genotype 1 and 2 sequences and found that only one genotype is present in each B19V-positive individual. We found that the B19V genotype 2 ITR regions, crucial for the virus replication, do not explain why genotype 1 overcame genotype 2 in circulation. Thus, factors that led to the disappearance of genotype 2 remain unknown.

One new research avenue emerging from our and others' recent work is archeovirology, i.e., *viruses in ancient human remains*. While viral genomic

prevalences in soft tissues have been investigated even from mummified human remains[195,263–265], no data of viruses persisting in human bone existed before 2015 when our Study III revealed B19V DNA in the bones of WWII casualties. This thesis concludes that human skeletal remains are an excellent source of viral genomes. In addition to B19V, we discovered 11 other human viruses in contemporary femoral bone, including several members of herpes- and polyomavirus families, as well as human papillomavirus 31, hepatitis B virus, and torque teno virus.

Soon after our WWII findings, another group described ten B19V sequences in ~500 to ~6900 years old dental and skeletal remains [12], and 12 hepatitis B virus genomes dating back 800 to 4500 years[239]. Most recently, they described variola virus sequences from the Viking Age[240]. Moreover, endogenous retroviral genomes have been analyzed from human skulls dated from the Copper Age to the 17th century[266]. As DNA, albeit fragmented, is most likely to be preserved across time in the hard tissues, ancient skeletal specimens will provide an unforeseen perspective on human pathogens and infectious diseases.

Moreover, *Bioportfolio*, holding the history of an individual's infectious encounters, can be of utility to anthropologists, archeologists, and forensic scientists. We disclosed with the two B19V genotype 3 positive WWII soldiers that virus genotyping can give perspectives on an individual's origin. Indeed, in addition to standard human DNA profiling, "Human Virome Panel" could be used in the future for the assessment of human provenance, to i) understand the population history and migrations and ii) identify unknown individuals or cadavers.

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