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Correlation of Neutralizing Tick-Borne Encephalitis Antibodies with the Immune Response to Yellow Fever Vaccination

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Abbreviations

Ab	Antibody
Abs	Antibodies
ADE	Antibody-dependent enhancement
CMC	Carboxymethylcellulose
DENV1	Dengue Virus Serotype 1
DENV2	Dengue Virus Serotype 2
DENV3	Dengue Virus Serotype 3
DENV4	Dengue Virus Serotype 4
DMEM	Dulbecco's modified Eagle medium
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediamine tetraacetic acid
EDII	Envelope protein domain 2
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FluoRNT	Fluorescence Reduction Neutralization Test
FRNT	Focus Reduction Neutralization Test
FSME	Frühsommer - Meningoenzephalitis
HIV	Human Immunodeficiency Virus
Ig-G	Immunoglobulin-G
Ig-M	Immunoglobulin-M
IIFT	Indirect Immunofluorescence Test
IRB	Institutional review board
JEV	Japanese Encephalitis Virus
MEM	Minimum Essential Medium
Nabs	Neutralizing antibodies
NEAA	Non-Essential Amino Acids
NT-TBEV	Neutralizing antibody titre against TBEV
NT90-TBEV	Neutralizing antibody titre against TBEV at 90% cut-off
NT80-TBEV	Neutralizing antibody titre against TBEV at 80% cut-off
NT-YFV	Neutralizing antibody titre against YFV
NT90-YFV	Neutralizing antibody titre against YFV at 90% cut-off
NT80-YFV	Neutralizing antibody titre against YFV at 80% cut-off
NS1	Non-structural protein 1
NS2A	Non-structural protein 2A
NS2B	Non-structural protein 2B
NS3	Non-structural protein 3
NS4A	Non-structural protein 4A

NS4B	Non-structural protein 4B
NS5	Non-structural protein 5
OAS	Original antigenic sin
PBS	Phosphat buffered saline buffer
PCR	Polymerase Chain Reaction
prM	Precursor protein M
protein C	Capsid protein
PRNT	Plaque Reduction Neutralization Test
protein E	Envelope protein
protein M	Matrix protein
RNA	Ribonucleic Acid
TBE	Tick-Borne Encephalitis
TBEV	Tick-Borne Encephalitis Virus
TBEV-PRNT	Plaque Reduction Neutralization Test for TBEV
WNV	West Nile Virus
YF	Yellow Fever
YFV	Yellow Fever Virus
YF17D	Live Attenuated Yellow Fever 17D Vaccine
ZKV	Zika Virus

Abstract in English

Due to global warming and advancing globalization, it is to be feared that flaviviruses will continue to spread. In the near future, this will result in intensified virological and infectiological research into the immune reactions to these viruses. A better understanding of the effects of pre-existing immunity on immune responses following vaccination is of great importance for translational infection research in order to develop more effective immunisation strategies. The Plaque Reduction Neutralization Test (PRNT) remains the methodological tool of choice to measure neutralizing antibody titres against Tick-Borne Encephalitis Virus (TBEV). In the present study, the neutralizing antibody titres against TBEV (NT-TBEV) before and 28 days after vaccination with the Live Attenuated Yellow Fever 17D Vaccine (YF17D) against the Yellow Fever Virus (YFV) were analyzed in comparison to the neutralizing antibody titres against YFV (NT-YFV), measured with the Fluorescence Reduction Neutralization Test (FluoRNT). In addition, the Indirect Immunofluorescence Test (IIFT) was used to test whether cross-reacting antibodies against other flaviviruses were present due to the previous TBE vaccination and how these behaved in comparison to TBE-naïve persons after the yellow fever vaccination.

It was shown that the administered yellow fever vaccine YF17D had no influence on pre-existing neutralizing antibody titres against TBE. Similarly, the yellow fever vaccination induced equally strong neutralizing antibody titres against yellow fever in TBE-immunised and TBE-naïve individuals. In addition, increased panflaviviral cross-reactivity in TBE-immunised individuals and the absence of cross-reactivity in TBE-naïve individuals following yellow fever vaccination was demonstrated. This resulted in the need to investigate further specific neutralizing and/or cross-reactive epitopes of flaviviruses. The presented work is an independent part of an article submitted for publication (1), which shows that vaccination against TBE and subsequently against yellow fever do not interfere with each other's robust immune response, but can intensify immunogenicity through antibody-dependent enhancement. The data suggest that yellow fever vaccination induces non-cross-reactive antibodies in flavivirus-naïve individuals, while enhanced cross-reactivities are present in TBE vaccinated individuals due to pre-immunity.

Abstract in German (Deutsche Kurzfassung)

Aufgrund der globalen Erwärmung und der fortschreitenden Globalisierung steht zu befürchten, dass sich Flaviviren weiter ausbreiten werden. Das wird in naher Zukunft eine intensivierte Erforschung der Immunreaktionen auf diese Viren zur Folge haben. Ein besseres Verständnis der Auswirkungen einer bereits bestehenden Immunität auf die Immunreaktionen nach einer Impfung ist von großer Bedeutung, um effektivere Immunisierungsstrategien zu entwickeln. Um neutralisierende Antikörpertiter gegen die durch Zecken übertragende Frühsommer-Meningoenzephalitis (FSME) zu messen, wurde der PRNT durchgeführt. In der vorliegenden Studie wurden die neutralisierenden Antikörpertiter gegen FSME (NT-TBEV) vor und 28 Tage nach einer Gelbfieber-Impfung mit dem attenuierten Lebendimpfstoff gegen Gelbfieber 17D (YF17D) im Vergleich zu den neutralisierenden Antikörpertiter gegen Gelbfieber (NT-YFV) analysiert, die mit dem FluoRNT gemessen wurden. Außerdem wurde mithilfe des IIFT getestet, ob aufgrund der vorhergehenden FSME-Impfung kreuzreagierende Antikörper gegen andere Flaviviren vorhanden waren und wie sich diese im Vergleich zu FSME-naiven Personen nach der Gelbfieber-Impfung verhielten.

Es konnte gezeigt werden, dass der verabreichte Gelbfieber-Impfstoff YF17D keinen Einfluss auf bereits vorhandene neutralisierende Antikörpertiter gegen FSME hat. Ebenso induzierte die Gelbfieber-Impfung gleich starke neutralisierende Antikörpertiter gegen Gelbfieber bei FSME-immunisierten wie bei FSME-naiven Personen. Darüber hinaus konnte eine erhöhte panflavivirale Kreuzreaktivität bei FSME-immunisierten Personen sowie das Fehlen einer Kreuzreaktivität bei FSME-naiven Personen nach einer Gelbfieber-Impfung nachgewiesen werden. Daraus ergab sich die Notwendigkeit, weitere spezifische neutralisierende und/oder kreuzreaktive Epitope der Flaviviren zu untersuchen. Die vorgestellte Arbeit ist eigenständiger Teil eines zur Veröffentlichung eingereichten Artikels (1), der zeigt, dass Impfungen gegen FSME und anschließend gegen Gelbfieber die jeweils robuste Immunantwort nicht gegenseitig behindern, sondern die Immunogenität durch antikörper-abhängige Verstärkung intensivieren können. Die Daten deuten darauf hin, dass die Gelbfieber-Impfung bei Flavivirus-naiven Personen nicht-kreuzreaktive Antikörper induziert, während bei FSME-geimpften Personen aufgrund der Vorimmunität verstärkte Kreuzreaktivitäten auftreten.

1. Introduction

Due to the warming of the climate with increased humidity, the living conditions for ticks and mosquitoes are constantly improving (2,3). Flaviviruses and other viral infections spread increasingly on a global scale and new pathogens emerge or can be regionally classified as emerging pathogens. Additionally, more and more vaccinations are available, and the overall population grows older. Thus, a rising number of individuals will have previous and potentially cross-reactive immune responses at the time of vaccination and/or natural infection, which could have many implications that remain largely unexplored.

Antibodies (Abs) have the ability to neutralize the virus by binding the viral envelope protein and thereby preventing the virus from entering the host cell. Neutralizing antibodies (Nabs) are regarded as the most important players of long-lasting protection after natural infection or vaccination against viruses such as flaviviruses.

As hypothesised by the original antigenic sin (OAS) model, a secondary immune response after exposure to a slightly varied antigen can possibly result in an unadapted/deficient immune reaction with worse clinical outcome. Cross-reacting immunoglobulins may also induce the so-called antibody-dependant enhancement (ADE) as reported for sequential dengue virus infections of different types and for live attenuated vaccination infection with thus increased immunogenicity. As another example, cross reactivity in serological diagnostic measures e. g. regarding flavivirus infection is well known.

Those phenomena need to be considered when performing diagnostics in routine patient care, talking about correlates of immunity, and/or investigating vaccinations as well as vaccination schedules. Inter-individual as well as inter-populational differences in immune responses to viral infections and vaccines are still poorly understood but dispose of increasing importance in the advancing era of personalised medicine.

Here, we report of influences of previous immunity against tick-borne encephalitis virus on immune responses as well as diagnostic assays following vaccination with the live attenuated yellow fever vaccine YF17D (YF17D) in healthy adults.

1.1. Flaviviruses

The family of Flaviviridae is a representative of the arboviruses, short for arthropod-borne viruses. The Flaviviridae family includes over 70 different virus types, which are assigned to four genera: Flaviviruses, Pestiviruses, Pegiviruses and Hepacicviruses (4).

Due to the jaundice it can cause in infected patients, the Yellow Fever Virus (YFV) gave its name to the family and genus of Flaviviridae/Flaviviruses: *flavus* in Latin means yellow (5). Apart from the eponym, several other human-pathogenic agents belong to the flavivirus genus, which use ticks or mosquitoes as vectors to infect both humans and animals.

Tick-borne viruses primarily infect rodents as natural hosts. However, the Tick-borne Encephalitis Virus (TBEV) may also cause an eponymous disease in humans (6).

Flaviviruses, which cause febrile sicknesses in humans and can be transmitted by mosquitoes, are divided into two groups: On the one hand, mosquitoes of the *Aedes spp*. are transmitting YFV or Dengue Virus (DENV). Another representative is the Zika Virus (ZKV), which also can cause infants to be born with microencephaly (7). These three viruses alone currently pose a major problem and risk to global health: Zika (2015 - 2016), yellow fever (2017, 2018) and dengue (2016) outbreaks emerged from many current endemic foci in recent years (8–10). On the other hand, *Culex* mosquitoes can trasnmit viruses such as St. Louis Encephalitis Virus, West Nile Fever Virus (WNV) or Japanese Encephalitis Virus (JEV), also causing neurological diseases such as meningitis or encephalitis in humans (11).

1.1.1. Viral structure

Flaviviruses have a diameter of 40 to 50 nm. The structural capsid proteins (protein C) form the capsid, which is enveloped by a lipid bilayer with two embedded structural protein types: Structural matrix (protein M) and envelope (protein E) proteins (Figure 1). The ribonucleic acid (RNA) genome consists of a positive sense single-stranded RNA and is located inside the capsid. The RNA of the flaviviruses has a large reading frame and translates the genome into a single common precursor polyprotein: it consists of both structural and non-structural proteins (12–14).

1.1.2. Viral proteins

1.1.2.1. Polyprotein

After infection of a host cell, the polyprotein represents the result of the translated viral RNA and is directly processed further during replication. The polyprotein comprises 3412 amino acids in the case of TBEV or 3411 amino acids in the case of YFV. Starting from the 5' cap, the sequences of the structural/non-structural proteins are found in the following order: protein C, viral membrane precursor product of protein M (prM), protein E and non-structural proteins 1-5 (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).

1.1.2.2. Structural proteins

Protein C, which consequently forms the capsid, interacts with the RNA genome due to its basic properties. This leads to the complexation of the RNA genome with protein C, the so-called nucleocapsid. (15–17). In the mature virion, protein M is anchored in the viral envelope membrane (12). The transitional state of heterodimeric complexes of PrM and E proteins functions as protection of the E protein from conformational changes induced by acidic pH. After cleavage of the PrM protein to Pr fragments and mature M protein, the E protein is released from the heterodimeric complex. The E protein can thereby form its homodimeric original form and fusogenic character again (18,19). Protein E is a glycosylated surface protein anchored in the viral envelope membrane. In addition to cell adsorption and entry into a target cell, its fusogenic property after endocytosis is crucial. After uptake into endosomal vesicles and exposure to low pH values, the conformation changes from 90 homodimers to 60 homotrimers. (19). This exposes the fusogenic domain of the E protein and triggers the fusion of viral membrane with endosome membrane (12). Furthermore, the E protein is mainly the target of the humoral immune response: Abs directed against the E dimer epitopes accessible in mature particles serve to neutralize the virus (20–23). These antibodies interfere with the fusion of virus and potential host cell, and can thus protect against reinfection with the same virus type (24). However, Abs are also formed against cryptic epitopes of the E protein that are not freely accessible in mature virions, such as the fusion loop at E protein domain 2 (EDII). Together with prM-specific Abs they are considered broadly cross-reactive

and form the basis of various immunological hypotheses, described in the upcoming chapters (11,23).

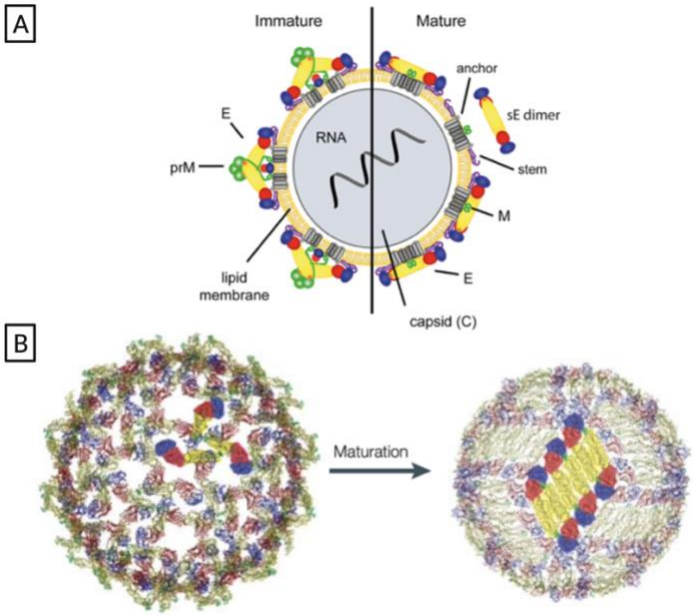

Figure 1. Visualisation of structural proteins in flaviviral virions.
(A) Schematic structure of immature/mature virus particles: The capsid formed by protein C contains the flavivirus genome and is protected by a lipid double membrane in which two other structural proteins are anchored: E protein, with its different domains (yellow/blue/red), enters into different configurations with the (pr)M protein (green) during maturation (reproduced from reference Vratskikh *et al.,* (2013) (25) under the CC-BY license). (B) Three-dimensional configuration of E-protein in immature and mature virion (reproduced with kind permission from Springer Nature and Mukhopadhyay *et al*., (2005) (5)).

1.1.2.3. Non-structural proteins

NS1 is the only non-structural protein resident in the lumen of the endoplasmic reticulum. High levels of soluble NS1 serve as correlate to the severity of disease (26–28). Both, NS1 and NS2A are involved in the maturation of new virus particles in addition to virus replication (29–32). NS2B acts as a cofactor for the NS3 serine protease. As a heterodimer with NS2A, NS3 represents a multifunctional protein: It serves as a serine protease, nucleosid-triphosphatase and helicase (33,34). NS4A has the task of initiating the curvature of the endoplasmic reticulum membrane. No enzymatic activities were yet uncovered for the NS4B protein. However, it interacts with NS3 and is therefore indispensable for virus replication (35). NS5 has an RNA-dependent RNA polymerase activity that replicates the viral genome (4,34,36).

1.1.3. Replication

Flaviviruses predominantly infect monocytes, macrophages and dendritic cells in affected patients (37–39). After adsorption to the cell surface, the virus enters the cell by receptor-mediated endocytosis (12). The low pH of the endosomal compartment triggers the conformational change of the homodimeric structure to a homotrimeric intermediate form of the E protein, which presents the fusogenic domain of the protein. This conditions the fusion of the endosome membrane with the viral membrane and thus the release of the nucleocapsid and RNA into the cytoplasm (40,41). Immediately afterwards the polyprotein is synthesised and further processed into its structural protein components: C, prM and E. Already during the synthesis of the polyprotein, transmembrane domains of prM and E proteins initiate its invagination (budding) (11). Especially the NS5/NS5B proteins, functioning as RNA-dependent RNA polymerases, the viral RNA is first replicated into a negative matrix strand and then into a new plus-stranded RNA genome (12). Viral assembly is initiated as the newly synthesised RNA genome is inserted into the previously described budding complex and thus incorporated by the C protein, which is enveloped by a lipid bilayer integrated with prM- and E-proteins, transiently complexing heterodimers. Trimers of prM- and E heterodimers give these now formed immature, non-infectious virions their spiky appearance (42). Encapsulation of immature particles from the rough endoplasmatic reticulum occurs and the particles are transported through the Trans-Golgi network. The Golgi-Apparat-resident protease furin causes the cleavage of the prM into pr-fragments and M-proteins and renewed remodelling of the E-proteins into dimers. This leads to the maturation of immature to now infectious virions (43,44). By exocytosis, pr-fragments and mature infectious virus particles are now secreted (45).

1.1.4. Yellow Fever Virus (YFV)

Yellow fever, which is endemic in tropical and subtropical regions of Africa, Central and South America is caused by the YFV and still represents a major threat to the public health to date (46). The sylvatic transmission cycle involves mosquitoes and various monkey species; humans are an accidental host (47). After infection of humans and following an incubation period of 3-6 days, a biphasic course of the disease can occur besides an asymptomatic course,

similar to a TBEV infection: In the initial phase, patients may usually experience flu-like symptoms. Far fewer patients develop severe complications after a short remission: High fever, jaundice, shock, and organ failure. About 50% of severe cases die from the infection (8,48,49).

The live-attenuated vaccine YF17D was introduced in 1937 (50). Officially approved for adults and children from 6 months, and with a single application promises lifelong immunization. Within 10 days 96% of vaccines and within 30 days, 99% develop protective vaccine protection (varying according to specific age groups) (51). After the initial onset of viraemia on days 5-7, immunoglobulin-M (Ig-M) develops with a lifespan of approximately 18 days. The subsequently formed immunoglobulin-M (Ig-G) persist for up to 40 years after vaccination (52). According to the WHO in 2016, a single vaccination means lifelong vaccination protection (8), but according to the RKI in Germany, a booster vaccination has been recommended since 2022 before travelling to endemic areas if the first vaccination is 10 years old or older (53). In addition to the detection of neutralizing antibodies as a correlate of vaccine protection after immunization, innate and T-cell mediated immune responses should not be underestimated (54–56). The vaccine interacts with antigen-presenting cells, such as dendritic cells, via multiple Toll-like receptors and generates a response of T-helping cells, which support and strengthen the humoral immune system (52).

Due to globalization and regional low vaccination coverage, outbreaks still occurred most recently four times in Brazil or Nigeria (2017 and 2018) (57). Approximately 200,000 severe cases are registered annually. Hence, YF vaccination is recommended for residents as well as travellers to endemic areas. In addition, apart from symptom-oriented therapy, there are no specific drugs available for patients targeting the infection (8,58).

1.1.5. Tick-borne Encephalitis Virus (TBEV)

Tick-borne encephalitis (TBE), induced by TBEV, plays a particularly important role in Europe. For the first time TBEV was isolated as the cause of life-threatening infections by Schneider in Austria in 1931 and five different subtypes were described to date (59): The European/Western, the Siberian, the Far Eastern, the Baikal and the Himalaya subtype. Main vectors for the European subtype are hard ticks of the genus *Ixodes*. The virus circulates

between vectors and small rodents or small mammals (as the main reservoir) on which infected ticks feed. Similarly, humans can serve as an accidental host. Hence, TBEV is mainly transmitted by tick bites. An alternative route of infection was described through unpasteurized milk from infected goats or sheep during viremia (60–62). While possible mother-to-fetus and aerosol infection is discussed, human-to-human infection is not known (63–65).

In endemic areas, especially in rural areas with much vegetation, it is assumed that about 0.1 - 5% of ticks are infected (66) and approximately 1 in every 100 - 300 tick bites causes disease (67). Although the number of reported cases fluctuates from year to year, only in Germany 535 cases were reported in 2020 (September 2020), about 14% more than in the comparable period of the previous record year 2018 (468 cases) (68). Up to 75% of infections are asymptomatic, with the remainder showing a biphasic course. The mostly asymptomatic incubation period of 7-14 days is followed by the viraemic/first phase with flu-like symptoms: fever, headache, fatigue and general malaise (69,70). After a symptom free period, a second phase may occur in one third of patients, with the risk of manifestations in the central nerval system such as meningitis, encephalitis, myelitis, or mixed forms. Mortality ranges from 1 - 40% (71), but only 1 - 2% for the European subtype (6). 10 - 20% of patients with symptoms in the central neurological system show long-term neurological damage (64).

In Germany, there are currently two licensed TBE vaccines available, based on purified, formalin-inactivated virions: FSME-IMMUN® is based on the TBEV strain Neudoerfl, first licensed in 1976. In contrast to the Encepur® vaccine based on the TBEV strain K23, which was authorised in 1991. Both can be used for adults as well as children older than one year. For both vaccines, there is a classical/conventional and a rapid vaccination schedule consisting of a basic immunization of three doses followed by booster vaccinations: Following the first dose, the second is administered within 1-3 months and the third dose within 5-12 months. Booster vaccinations are given after 3 years and then repeatedly at intervals of 5 years. The efficacy of a prophylactic immunization against TBE, with a side-effect profile typical of vaccinations, is convincing. After the basic immunization, it is assumed that there is complete vaccination protection, which should last at least 3 years. In a study by Plentz *et al.,* (2009), a protective antibody titre was still found in 99% of the 19- to 51-year-old participants 5 years after basic

immunization and the first booster with Encepur® (72). In the study by Wittermann *et al.*, (2015), protection was present in 100% of participating children (73). Similarly, the protective antibody titre after FSME-IMMUN® vaccination with a comparable study design (basic immunization and one booster) was 93%/89% after 7/10 years in 18- to 49-year-olds, 82%/75% in 50- to 60-year-olds and 50%/38% in >60-year-olds (74). Overall, TBE vaccines show great protection against homologous and heterologous subtypes but provide no cross-neutralization against other flaviviruses (75,76). Despite the milestone of efficient vaccinations, TBE infections continue to increase as a result of low vaccination coverage (71).

As there is still no causal therapy for TBE, only symptom-oriented treatment is possible (77). Although an immunotherapeutic approach with monoclonal antibodies seems auspicious (78,79), in Europe this therapy was discouraged because ADE represents too great a risk (80,81).

1.2. The original antigenic sin (OAS)

The original antigenic sin (OAS) hypothesis was already described for various viruses: the influenza virus, human immunodeficiency virus (HIV) or flaviviruses such as DENV and the closely related ZKV. The hypothesis attempts to describe, that individuals form antibodies against viral epitopes after an initial infection and do not produce new specific antibodies in a subsequent infection with slightly varying epitopes. The antibodies of the initial infection usually suppress the production of specific antibodies by naïve B cells against the new virus, which would initiate a faster and more competent immune response. Instead, the immune system does not adapt and relies on the antibodies of the previous infection, which, however, have a poorer specificity and consequently weaker effectiveness against the new virus. The advantage of this mechanism would be the faster reaction of B cells with antibodies from the initial infection, whereas the disadvantage predominates, as no specific antibodies formed against the new virus. (82–85)

As already mentioned, OAS was particularly described for DENV and ZKV: Both viruses are transmitted primarily by mosquitoes of the genus *Aedes*, although Zika virus can also be passed congenitally or perinatally from mother to child. Overall, DENV has four main serotypes (DENV 1-4) and infection can lead from self-limiting dengue fever to dengue

haemorrhagic fever or dengue shock syndrome. As early as 1983, the OAS was identified in DENV and severe disease was linked to secondary infection with another serotype (86,87).

In the context of OAS, ZKV and DENV show more than 55% homology in the amino acid sequence of their E-proteins (Figure 2). This leads to the hypothesis that the phenomenon of severe disease in a ZKV infection could occur after a previous DENV infection (82). The already existing antibodies have the potential of cross-reactivity, which in the worst case can lead to antibody-dependent enhancement (88,89).

1.3. Neutralizing antibodies and cross-reactivity

As already described, different antibodies of the humoral defence dominate the response to an infection with flaviviruses: anti-E, anti-prM and anti-NS1 Abs. Virus neutralization is mostly completely accounted for by antibodies against epitopes of the E protein, which are accessible in mature virions. These follow the principle of 'multi-hit', as well as 'coating'. This means that in addition to interfering with receptor-mediated cell entry or preventing membrane fusion in the endosomes, the docking of a certain number of Nabs to the virus, as well as cross-linking of these virus-Nab complexes, also leads to sufficient neutralization. (23,90–92)

The representatives of the Flaviviridae family are antigenically related and there are great structural similarities, thus, multiple infections with representatives of the flaviviruses were strongly associated with serological cross-reactivity (93,94). Besides cross-reactive prM-specific Abs, specific cross-reactive sites were detected on E proteins of different flaviviruses by using monoclonal antibodies (95–100). The fusion loop at the tip of domain 2 is the most dominant element of the E protein, considered highly conserved within the family and the most exposed protein against which cross-reactive antibodies are formed in the course of an immune reaction with flaviviruses (23,101). The property of cross-neutralization among closely related viruses causes the association of individual representatives of the flaviviruses into serocomplexes, while the relations within as well as between serocomplexes can be very heterogenous and cross-protection becomes less likely among more distant flaviviruses (23,45,102,103). As shown in the dendrogram below (Figure 2), YFV and TBEV share only around 40% of the same amino acid sequence of the E-protein, while DENV and ZKV show more than 55% congruence.

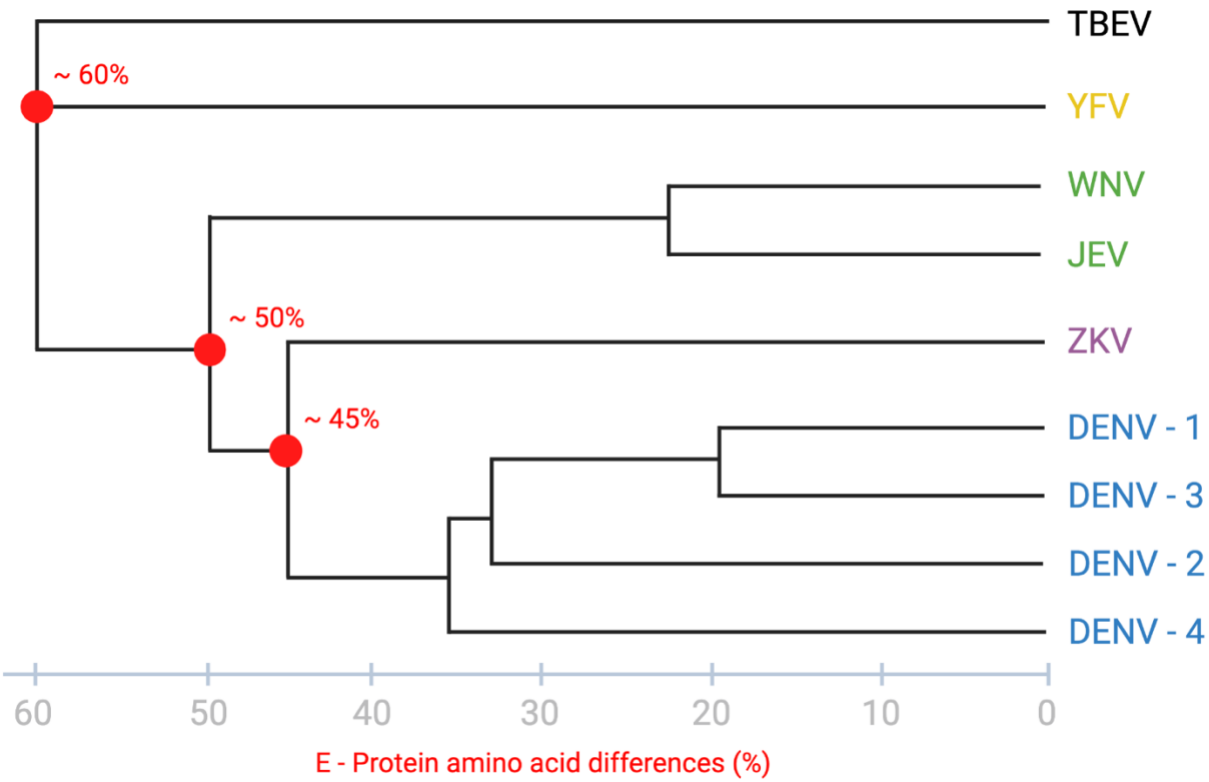

Figure 2. Antigenic relationships between flaviviruses.
The differences of the most important human-pathogenic flaviviruses are shown in percentages of divergent amino acids between their E proteins. TBEV (black) and YFV (yellow) share approximately 40% of the same amino acid sequence (modified and reproduced with kind permission from American Society for Microbiology and Heinz & Stiasny *et al.,* (2017) (45)).

Besides false-positive results as an immediate consequence (104), cross-reactive antibodies also serve to protect the host if they are present in sufficient quantities and hold high specificities (Figure 3) (105,106). Not long after the TBE vaccine was introduced in the 1970s, the amplification of cross-reactive immunoglobulins was observed in a TBE vaccination study with YF-pre-vaccinated individuals. Besides, in six out of nine subjects boosted YFV titres could be evoked after the first TBE vaccination, also low titres of heterotypic neutralizing antibodies against DENV2 could be detected (107). In another study, increased JEV neutralizing antibody titres were detected with pre-existing TBE vaccine-derived protection after the first shot with an inactivated JE vaccination candidate (108). Individuals vaccinated against JEV, TBEV and YF17D also showed neutralizing antibody titres against WNV, although those who lacked only the YF17D vaccination had fewer and lower neutralizing antibody titres against WNV. Neutralizing antibody titres against DENV2 showed similar results, regardless of whether

YF17D vaccination was given or not (109). As detected by Houghton-Trivino *et al.,* (2008) more than 80% of DENV patients showed highly neutralizing antibodies against YF (NT-YFV) (110). Conversely almost no cross-reactivity was found by Souza *et al.*, (2019), as samples with NT-YFV revealed nearly no detectable activity in DENV or ZKV Enzyme-linked Immunosorbent Assay (ELISA) Ig-G test kits (111).

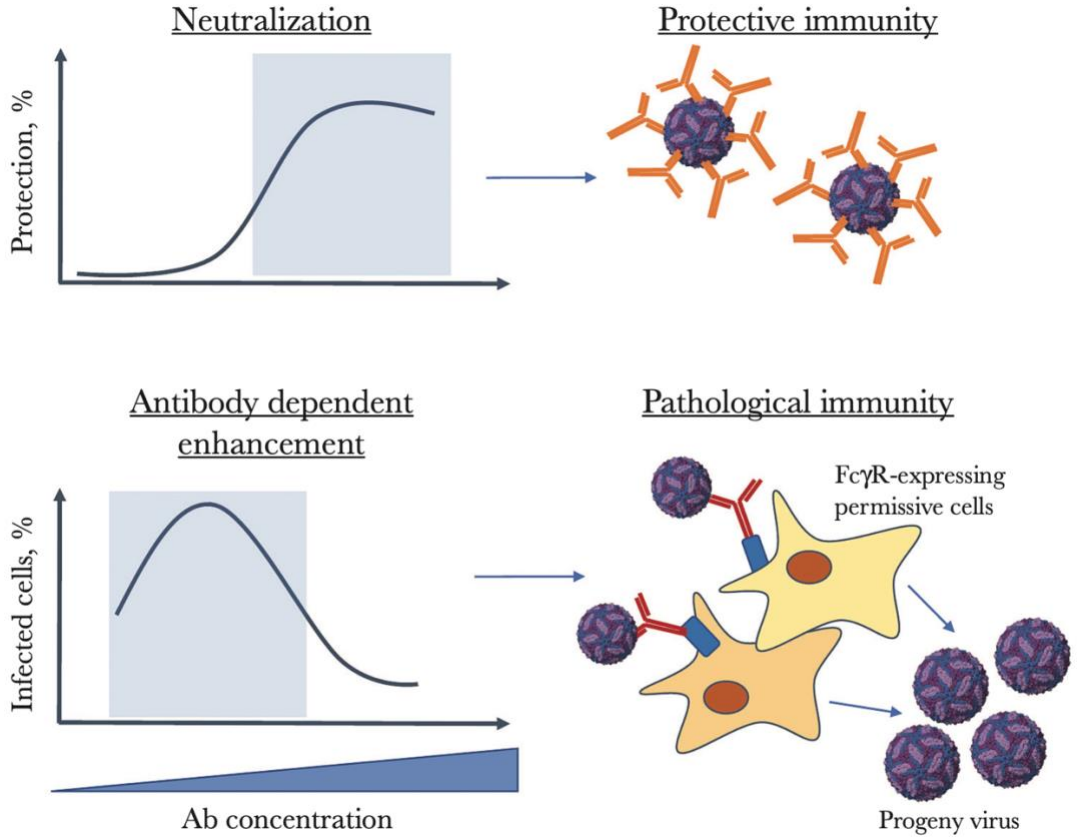

Figure 3. Cross-neutralization or ADE due to cross-reactive Abs.
As shown in the first row, at a certain threshold, cross-reactive Abs can serve to neutralize the virus sufficiently and protect the host. However, if the concentrations fall and decrease in specificity (second row), cross-reactive Abs are no longer protective, and ADE resulting in another viral replication site is a threatening consequence for the host (reproduced with kind permission of Oxford University Press and Priyamvada *et al.,* (2017) (112)).

However, if there is an insufficient amount and low specificity of cross-reactive antibodies, they can lead to failing of cross-neutralization: For instance, studies in hamsters even indicated that prior vaccination against YFV resulted in less protection against subsequent WNV infection than prior JEV immunization, again pointing out the importance of the arrangement of serocomplexes (Figure 2), as WNV and JEV share the same serocomplex (113,114).

A close connection to the focus of the here presented work was pointed out by Bradt *et al.*, (2019): After TBEV immunization, a YF pre-vaccinated group showed significantly lower TBEV neutralizing antibody titres than a flavivirus-naïve group, whereas YF neutralizing antibody titres did not alter significantly. Cross-reactive antibodies, which were tested exemplarily for DENV1, showed strong boosts in both groups after completing the vaccination schedule (115).

The worst case scenario of insufficient amounts and low specificities of cross-reactive antibodies is the initiation of antibody-dependent enhancement (ADE) and consequently promoted diseases because of no neutralization at all (11,23,106).

1.4. Antibody-dependent enhancement (ADE)

Like the OAS model, the ADE, which is basically based on the OAS and triggered by antibodies, was observed with various viruses: DENV, WNV, ZKV, Influenza A, Ebola, or HIV. For dengue, infection with different serotypes is responsible for ADE.

Since no more specific antibodies are formed in addition to cross-reactive, sub-neutralizing immunoglobulins, the humoral immune response is already weakened at the beginning of a secondary infection with another serotype as in the primary infection. The antibodies bind the virus but cannot neutralize it. Nevertheless, these complexes are phagocytosed by antigen-presenting cells (e.g., macrophages and B-cells (116,117). The virus thus generates another reservoir for viral replication, leading to a massively increased viral load in the second infection and, using the example of DENV, potentially resulting in dengue haemorrhagic fever or dengue shock syndrome (Figure 4) (118–120).

ADE is therefore also a major problem in the development of live vaccines which, for example, was shown in the recent development of a dengue vaccine (121). The aim of vaccines against flaviviruses is therefore to achieve sufficiently high titres of high affinity, low-cross-reactive antibodies. The TBEV and YFV vaccines both provide robust protection without the evidence of ADE.

Although ADE is equally not considered a major threat to the YF17D vaccine. Exact immunological mechanisms and why the vaccine is so potent is not yet fully understood. In a study by Vratskikh *et al.*, (2013) with subjects without a previous TBE vaccination and no

detectable neutralizing antibodies against TBEV (NT-TBEV), the level of the YFV titre in addition to the subclasses of antibodies against YFV were examined more closely (25). In summary, specific immune responses following YF17D vaccination were highly variable but suppose high neutralizing and low cross-reactive antibody titres.

Furthermore, only recently in a study by Chan *et al.* (2016), groups were administered the inactivated JE vaccine and later the live YF17D vaccine at three different time intervals (120). The control group were vaccinated against YF without prior JE vaccination. It was shown that certain amounts of cross-reactive immunoglobulins, induced by prior JE vaccination, enhance immunogenicity after YF vaccination by causing a prolongation of viremia and stronger proinflammatory responses (120). This indicates that ADE should not be disregarded.

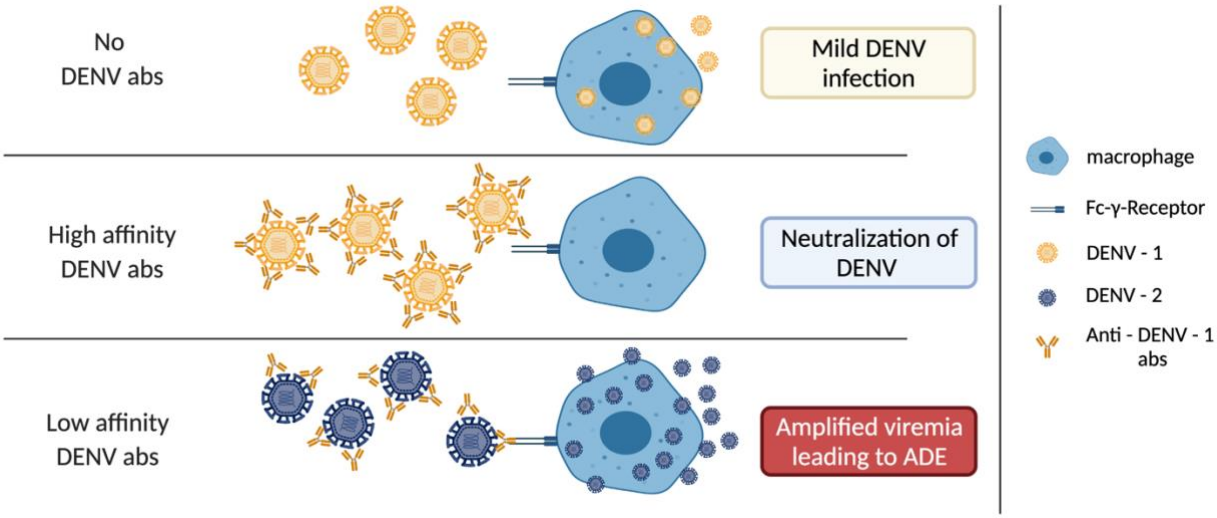

Figure 4. Antibody-dependent enhancement.
This diagram shows three alternative pathways of dengue virus infection, exemplary for flaviviruses: (Upper Row) There exists no immunity and a primary infection with a mild course occurs. (Middle Row) Due to an existing immunity, there are already sufficiently many and high affinity antibodies, which can completely neutralize the virus in a subsequent infection. (Bottom Row) There was contact with flaviviruses, but pre-existing antibodies are quantitatively and qualitatively deficient in an antigenically similar, slightly varying serotype. Since they bind but do not neutralize the virus, the phagocytosis of antibody-virus complexes creates another replication site for the virus. Thus, a secondary (dengue) infection may lead to a much larger viral load, which subsequently may result in a severe clinical outcome (modified and reproduced from reference Izmirly *et al.,* (2020) (101) under the CC-BY license).

1.5. Diagnosis of flavivirus infection

To reliably determine a flavivirus infection, the interaction of different components should be considered: Possible exposure, specific symptoms, pathologically altered blood parameters and a direct or indirect virus detection. Since symptoms of infection range from absent to flu-like up to inflammatory symptoms of the cerebral nervous system, this sometimes complicates the diagnosis of choosing the right testing procedure (122). Consequently, the choice of diagnostic tests should be adapted to the patient's medical history and selected carefully, depending on and how long ago a suitable exposure occurred and how long symptoms were present.

In general, infection with either TBEV or YFV clinically often occur with a biphasic clinical course. In the initial phase non-specific flu-like symptoms may emerge, which are associated with viremia. In this case the virus can directly be detected in the patients' blood by using Polymerase Chain Reaction (PCR) or the older and no longer primarily used method of virus isolation. After most patients subsequently enter the convalescence phase, a few, however, experience the second phase with organ-specific manifestations after a short period of symptom-free conditions. Thus, in the second phase with a possible severe clinical course, but also in convalescents or vaccinated persons, indirect detection of the infection may be possible with the aid of the identification of Ig-M) and Ig-G by screening method ELISA or Indirect Immunofluorescence Tests (IIFT), as well as neutralizing antibodies in Plaque Reduction Neutralization Tests (PRNT). In addition, different possibilities of indirectly detecting an infection are complement fixation assay and hemagglutination inhibition tests (123,124). As known, flaviviruses harbour a high potential of cross-reactive antibodies among themselves whether a natural infection or vaccination occurred. This characteristic of the antibodies can be observed well in ELISA or IIFT, but not in neutralization tests. If cross-reactivity can be detected, a previous contact with flaviviruses could be assumed and a supposedly different clinical picture in terms of modified immune responses may be observed (125).

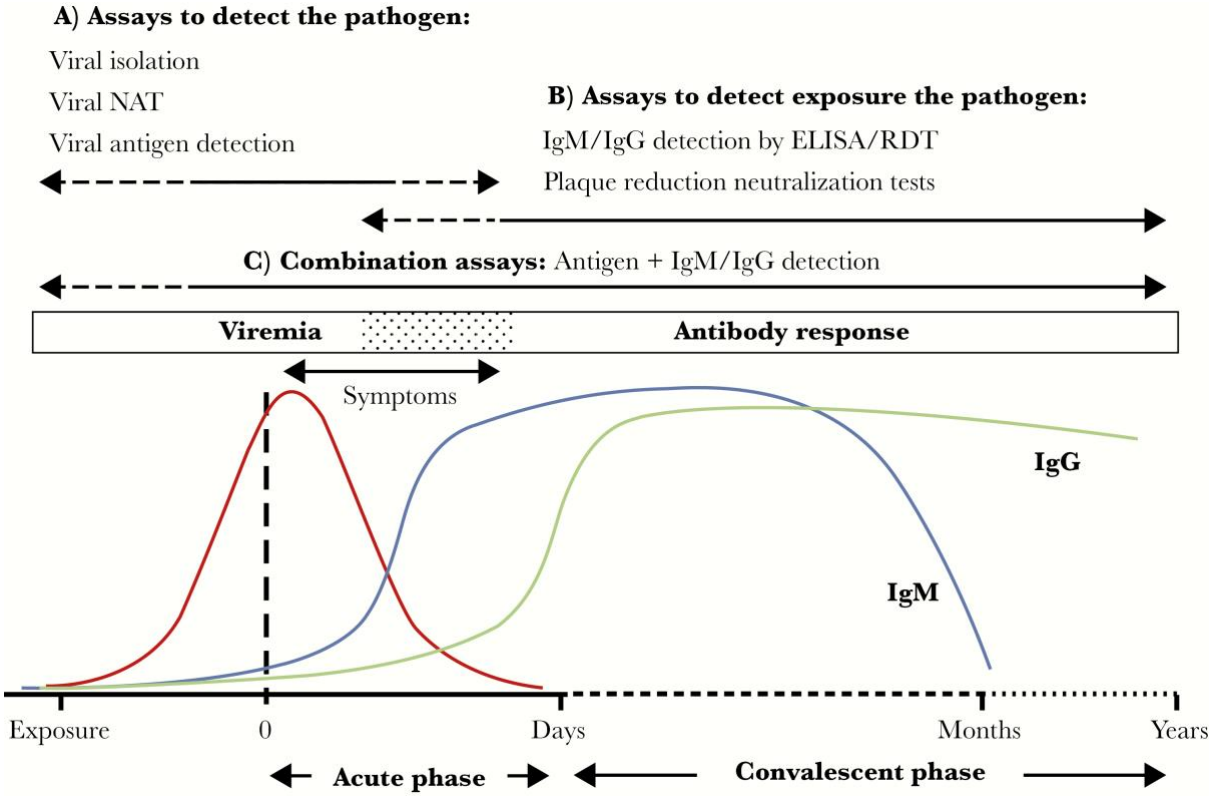

Figure 5. Time course of flavivirus infection and diagnostic means to confirm infection.
Infection with a flavivirus is often biphasic. Only the first phase is shown here, in which the peak of viraemia ("0") approx. 3-5 days after exposure heralds the acute phase. It can cause symptoms and is also the approximate time of onset of the adaptive immune system with antibody formation as the immunological response to the pathogen. From the time of exposure until the pathogen clears the bloodstream within days, virus, viral genome, and viral antigens can be detected directly by nucleic acid amplification tests (NAT) such as PCR or virus isolation. As soon as primary Ig-M and secondary Ig-G antibodies are formed, it is possible to detect them in ELISA / IIFT (not shown) and to assess their neutralizing quality in PRNT (reproduced with kind permission of Oxford University Press and Goncalves *et al.,* (2017) (126)).

1.6. Objectives

While more and more vaccinations against flaviviruses are becoming available and spontaneous infections with Flaviviruses increase, it seems likely that more frequently people with existing flavivirus immunity are being vaccinated.

In this context, the aim of the presented doctoral thesis was the investigation of the influence of pre-existing neutralizing TBE antibodies (on day 0) on the immune response following YF17D vaccination. Whether the neutralizing antibody titre against TBEV (NT-TBEV) varies from before to after YF17D vaccination (e.g., due to a possible bystander effect or cross-reactivity/immunoactivation) or remains unchanged, should be investigated by determining NT-TBEV at baseline and 28 days after YF17D vaccination.

In the second part of this thesis, the Indirect Immunofluorescence Test (IIFT) was being used to examine the development of broadly flavivirus cross-reactive antibodies between eight different flavivirus infections (Yellow Fever Virus, West Nile Virus, Tick-borne Encephalitis Virus, Japanese Encephalitis Virus and Dengue Virus I-IV) following YF17D vaccination in subjects with and without pre-existing TBE vaccination protection, in each case on day 0 and day 28.

The determination of neutralizing antibody titres against YFV (NT-YFV) 28 days after YF17D vaccination was investigated by Lisa Lehmann, a doctoral student of Prof. Rothenfusser at the Department of Clinical Pharmacology of University Hospital LMU Munich and were used with kind permission for these analyses.

2. Experimental Section

2.1. Materials

2.1.1. Instruments

Device	Modell	Manufacturer
Incubator cells	Incubator CO2 Galaxy 170 S	Eppendorf New Brunswick (Hamburg, Germany)
	Heraeus BBD 6220 CO2 Incubator	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
Incubator plates	Heraeus Kendro HeraCell 150 CO2 Incubator	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
Heater	Typ WB 10	P-D Industrial society mbH (Dresden, Germany)
Table incubator	ThermoMixer® C	Eppendorf (Hamburg, Germany)
Counting chamber	Neubauer Improved DHC-N01	NanoEnTek (Seoul, Korea)
Shaker	Minishaker MS1	IKA-Werke (Staufen, Germany)
	Minishaker MS3 digital	
	Lab dancer	
Sterile safety cabinet	Claire® pro	Berner (Elmshorn, Germany)
Safety cabinet	Herasafe KS	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
Inverse microscope	DM IL LED	Leica Microsystems (Wetzlar, Germany)
Washing automat	MERGITE! 10	EUROIMMUN AG (Lübeck, Germany)

2.1.2. Cultured human cell lines

Cell line	Manufacturer
A549 (ATCC® CCL-185™)	American Type Culture Collection (Manassas, VA, USA)

2.1.3. Chemicals

Chemical	Manufacturer
Trypsin- Ethylenediamine tetraacetic acid (EDTA) (0.05%, phenol red)	Gibco, Thermo Fisher Scientific Inc. (Waltham, MA, USA)

2.1.4. Indirect Immunofluorescence Test (IIFT) - Kits and equipment

Product	Manufacturer
Flavivirus Mosaic 3 Ig-G-Kit	EUROIMMUN AG (Lübeck, Germany)
Zika-Viruses (ZIKV) Ig-G-Kit	
FITC-labelled Anti-Human Ig-M and Ig-G	
Ig-M-positive control against DENV	
EUROSORB	

2.1.1. Buffers, media and solutions

Buffers	Concentration & Chemical	Manufacturer
Washing buffer	Phosphat buffered saline (PBS) buffer (1X, Dulbecco's) – Powder, pH: 7.5 ± 0.2	PanReac, AppliChem GmbH, ITW Reagents
Cell culture medium	Minimum Essential Medium (MEM) + GlutaMAX™	Gibco, Thermo Fisher Scientific Inc. (Waltham, MA, USA)
	1x Minimum Essential Medium (MEM) non-essential amino acids (NEAA) (100x)	
	10% Fetal Bovine Serum (FBS)	
Dilution medium	MEM + GlutaMAX™	Gibco, Thermo Fisher Scientific Inc. (Waltham, MA, USA)
	1x MEM NEAA (100x)	
CMC medium	MEM + GlutaMAX™	Gibco, Thermo Fisher Scientific Inc. (Waltham, MA, USA)
	1x MEM NEAA (100x)	
	2% Fetal Bovine Serum	
	0.75% Carboxymethylcellulose (CMC)	Sigma-Aldrich (St. Louis, MO, USA)
Staining solution	0.1% Crystal Violet	Sigma-Aldrich (St. Louis, MO, USA)
	13% Formaldehyde solution	Sigma-Aldrich (St. Louis, MO, USA)

2.1.2. Consumables

Consumable	Manufacturer
Nunc™ EasYFlask™ 75cm^2, 175 cm^2	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
24-well cell culture well plate	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
	Greiner Bio-One (Frickenhausen, Germany)
96-well cell culture well plate Costar ®	Corning Incorporated Life Sciences (Tewksbury, MA, USA)

2.1.3. Software

Software
Graphpad (Prism Version 9)
Microsoft Excel Version 16.47.1
Microsoft Powerpoint Version 15.52
Biorender.com (Free Version)

2.2. Methods

The analytic work regarding the Plaque Reduction Neutralization Test for TBEV (TBEV-PRNT) was carried out in the BSL3** laboratory of the Bundeswehr Institute for Microbiology in Munich under the guidance/supervision of PD Dr. Dobler. IIFTs were carried out in cooperation with the laboratory of the Institute for Clinical Chemistry at the University Hospital rechts der Isar, Technical University Munich.

2.2.1. Study cohort, participants and ethical approval

The establishment of the vaccination cohort was covered by funding from the iMed consortium of the Helmholtz Society to Prof. Rothenfusser (duration 2015-2019; titled "Yellow fever vaccination" and "Influenza immunization in the elderly" cohort studies). Ethical permission of the responsible institutional review board (IRB) at the Medical Faculty of Ludwig-Maximilians-Universität Munich was granted prior to study initiation (424-15 and 86-16). The clinical study was retrospectively registered in the ISRCTN registry (https://doi.org/10.1186/ISRCTN17974967). From 2015 until 2019, a study cohort with a total of 250 participants was recruited. Study participants were vaccinated subcutaneously with the YF17D vaccine (Stamaril; Sanofi Pasteur, Lyon, France) and blood, serum, urine, saliva, and stool was sampled before, and on days 3, 7, 14 and 28 after YF17D vaccination. Serum and plasma samples were stored at -80°C. Study participants were healthy adults naïve to natural flavivirus infection and with a negative vaccination history in regard to JEV and YFV. Overall sodium-heparinised samples from 242 of the 250 recruited vacinees were available at the time this study was performed, and could be included into this study (Figure 6).

In the following, study participants with self-reported history of TBE vaccination more than 4 weeks prior to study inclusion will shortly be called TBE vaccinated. Subjects without self-reported history of TBE vaccination will be called TBE non-vaccinated**.**

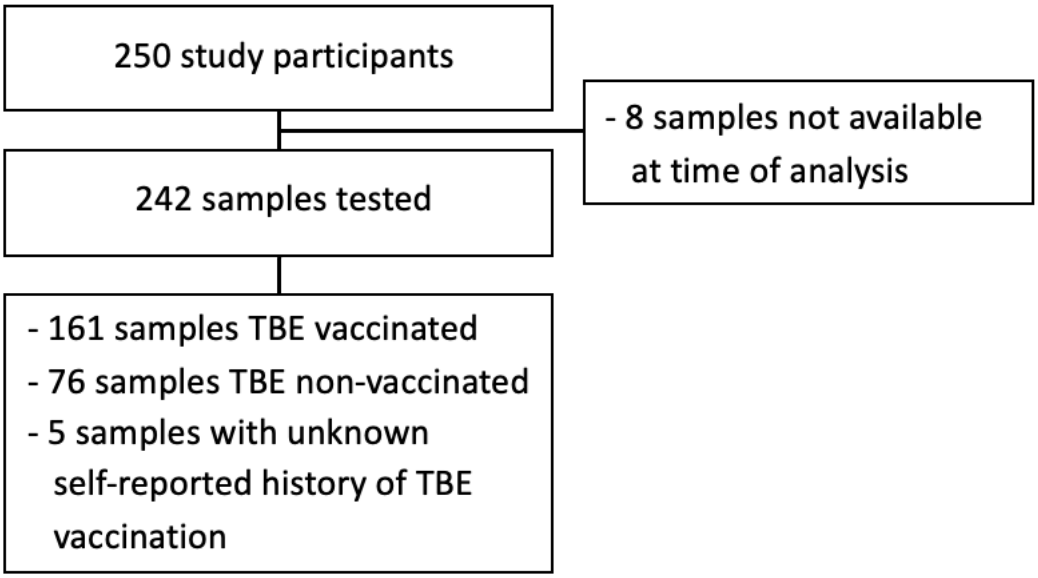

Figure 6. Flowchart of study cohort and included study participants.

2.2.2. Plasma samples

Samples and associated personal information used in this study were originated from the presented YF vaccination cohort study that was conducted from 2015 – 2019 at the Division of Infectious Diseases and Tropical Medicine (DIDTM) as well as the Department of Clinical Pharmacology, University Hospital, LMU Munich, Germany. Samples were analyzed retrospectively and in an pseudonymized manner. After giving informed consent, blood samples used here were collected directly before as well as on days 3, 7, 14 and 28 after administration of YF17D (0.5 mL of Stamaril; Sanofi Pasteur, Lyon, France) subcutaneously at the DIDTM. Blood samples were taken by phlebotomy and sodium-heperanized plasma samples were aliquoted and stored at -80°C until analysis by neutralization tests or IIFTs (Figure 7).

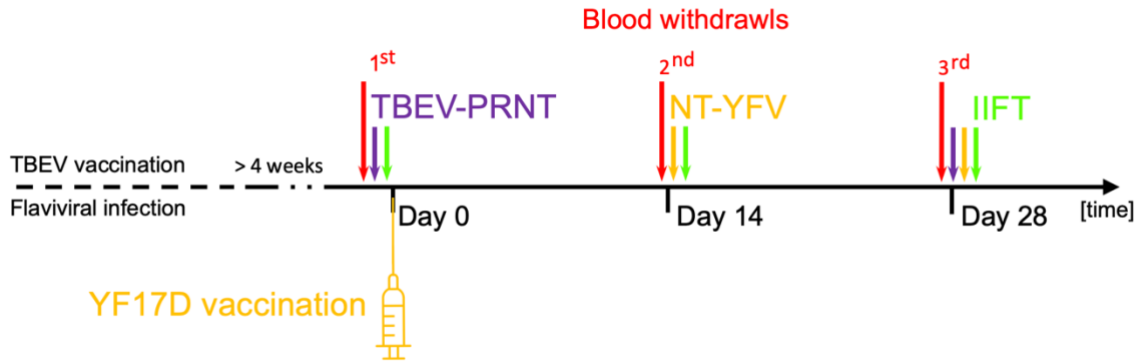

Figure 7. Study timeline.
For inclusion in study, history of natural infection with flaviviruses and TBEV vaccination was determined. Immediately after first blood draw on day 0, YF17D vaccination was administered. TBEV-PRNT (purple arrow) and IIFT (green arrow) were performed on samples from day 0 and 28, NT-YFV (yellow arrow) was performed on samples from day 14 and 28 after YF17D vaccination.

2.2.3. Virus stock

We used the TBEV reference strain Neudoerfl (127). The strain was initially passaged two times on A549 cells (MEM, 2 % FBS, 1x NEAA, 4 days) to produce enough concentrated virus stock solution (MEM, 20 % FBS, 1x NEAA), which was subsequently kept at -80 °C. The virus titer (1.5 x 10^7 pfu/ml) of the stock solution was analyzed by performing a PRNT with ten-fold dilutions and calculated according to (128).

2.2.4. Cell culture

All materials were used in sterile cell culture in a sterile workbench, treated accordingly and contaminated material was promptly and properly disposed of. All liquid materials were heated to 37 °C before use/contact with cells. A549 lung adenocarcinoma cells (ATCC® CCL-185™) were cultivated as confluent monolayer (MEM, 1x NEAA, 10 % FBS) in an incubator at 37 °C and 5 % CO_2, where they grew on the surface of the bottom of T-75/ T-175 cell culture bottles. After the formation of a confluent unicellular layer (cell monolayer) with at least 90% confluence, the bottom of T-175 cm^2 bottles contained approx. 2.5 x 10^7 cells. Under the conditions described above, the number of A549 cells doubled approximately every 24-36 h. About every second to third day the cells were split after forming a confluent monolayer and the medium was renewed.

For splitting, the entire cell culture supernatant was poured off and washed twice by adding and pouring off 10 mL PBS. By adding and immediately pouring off 5 ml/bottle of trypsin-EDTA solution and incubating for 5 - 10 minutes in the cell culture incubator with another 3 ml/bottle of trypsin-EDTA solution, the adhesion proteins of the cell surface were gradually cleaved. The cells could now be easily detached from the substrate by gently swivelling them horizontally and cautiously tapping the side of the bottle. After all cells were detached from the bottom, controlled by sight under the microscope, the trypsin-EDTA was inactivated by adding 10 ml of medium. Subsequently, all cells and medium were collected, resuspended, split and filled up with medium so that 20ml/bottle were again incubated in 5% CO_2 atmosphere at 37 °C.

2.2.5. Plaque Reduction Neutralization Test for TBEV (TBEV-PRNT)

The PRNT is considered the gold standard in the determination of NT-TBEV. A protocol based on Litzba *et al.*, (2014) was developed in the laboratory of Dr. Gerhard Dobler at the Bundeswehr Institute for Microbiology in Munich, Germany, and was considered the most suitable (124). Regarding the laboratory workflow (Figure 8): First, the cohort was sorted according to their self-reported vaccination history: There were n=161 TBE vaccinated, n=76 TBE non-vaccinated and 5 study participants whose vaccination history remains unclear were declared as 'unknown', as they were not sure if they have been vaccinated against flaviviruses or not. In the case of vaccinated persons and those declared as 'unknown', the NT-TBEV was determined on day 28 and on day 0. In the case of non-vaccinated persons with a NT-TBEV on day 28, the NT-TBEV was determined on day 0, but if no NT-TBEV could be determined on day 28, no titre determination was carried out on day 0.

First, the NT-TBEV that resulted in 90% a 90% inhibition of virus replication (NT90-TBEV), in all subjects on day 28 were analyzed. Thereafter, the NT90-TBEV on day 0 in all participants with detectable NT90-TBEV (≥ to 1:10) on day 28 were determined. However, if there were discrepancies between the vaccination history and the titre determined on day 28, e.g., without self-reported history of TBE vaccination and detectable NT90-TBEV on day 28 or with self-reported history of TBE vaccination and no NT90-TBEV on day 28 (≤ 1:10), the respective NT90-TBEV on day 0 were also analyzed.

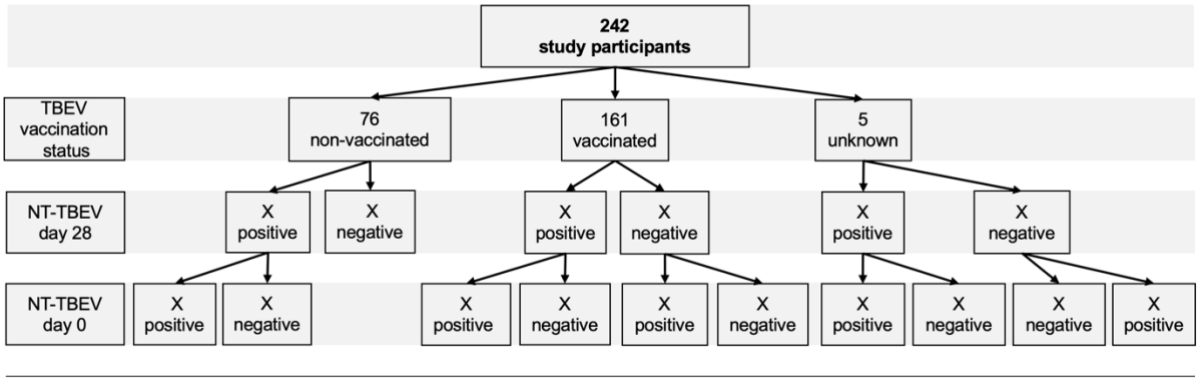

Figure 8. Workflow of TBEV-PRNT.

Workflow of TBEV-PRNT of study participants' plasma samples before (day 0) and after (day 28) vaccination with YF17D. Positive: NT90-TBEV was detectable at a titre of ≥ 1:10 in TBEV-PRNT, negative: no NT90-TBEV was detectable at a dilution of 1:10 in TBEV-PRNT.

2.2.5.1. TBEV-PRNT test protocol for 24-well plates

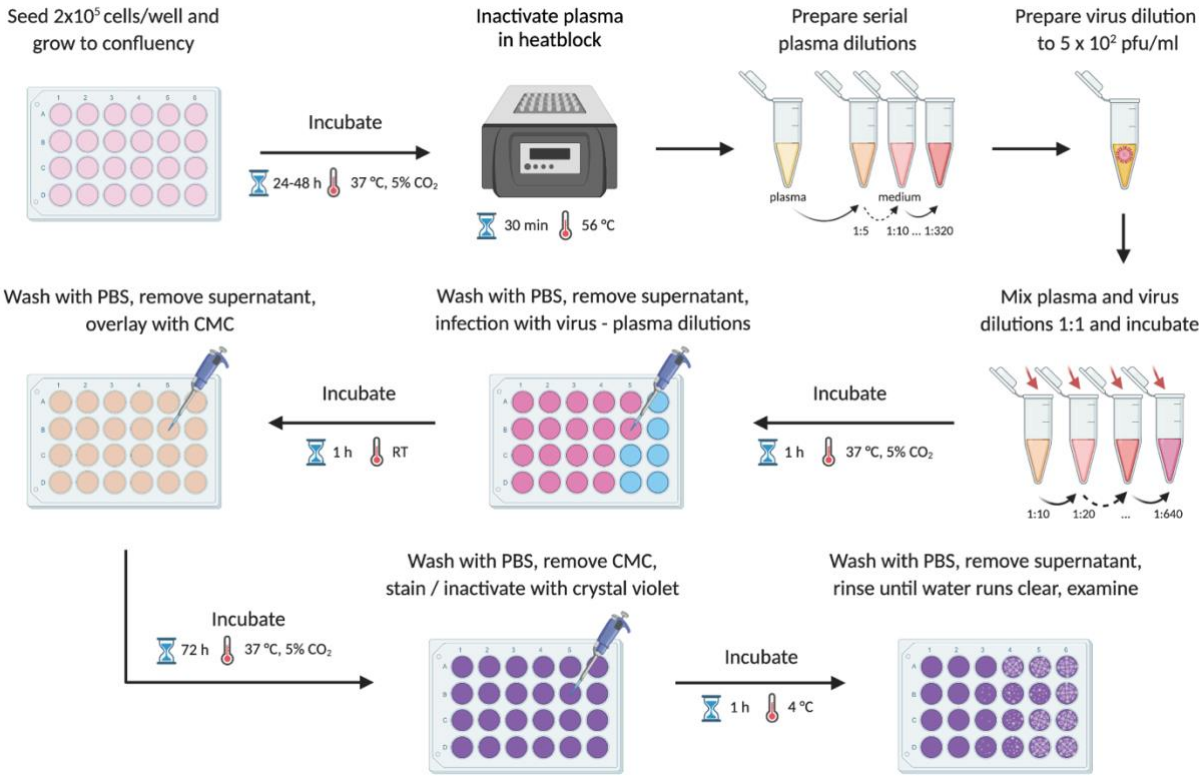

Figure 9. TBEV-PRNT protocol for 24-well plates.

A549 cells were cultured in 24-well plates for 24 to 48 hours to achieve confluent monolayers. After 30 minutes of inactivation of plasma samples at 56°C, a sequence of seven dilutions (from 1:5 to 1:320) was prepared. Since further pathogens and components of the complement system were removed by the inactivation of the plasma, the activity of the antibodies to be investigated, i.e. the virus neutralization, could be observed without external influences (129–131). The virus aliquot was thawed at room temperature, thoroughly mixed, and diluted to a concentration of 500 pfu/mL in a biohazard safety hood. Equal volumes of plasma and virus dilutions were mixed and incubated for 60 minutes at 37 °C and 5 % CO_2. Then the cells were rinsed with PBS and inoculated with 100 µL of virus-plasma mixture in triplicates. On each plate positive and negative controls were performed in triplicates. After the cells were incubated for 60 minutes at 37 °C and 5 % CO_2 and washed with PBS, 500 µL CMC medium (0.75 % CMC, MEM, 2% FBS, 1x NEAA) was added. The 24-well plates were incubated for three days at 37 °C and 5 % CO_2. The supernatant was discarded, the assays were coated and dyed with crystal violet (0.1 % crystal violet, 13% formaldehyde) at 4 °C, washed and dried for visual examination. The plaques of each well were counted on a light panel (Figure 9) (1).

2.2.5.2. TBEV-PRNT test protocol for 96-well plates

The same protocol (Figure 9) was transferred to 96-well plates to generate a temporarily higher turnover. For this, volumes were adapted to the respective substrate: A549 cells were cultured in 24-well plates for 24 to 48 hours to achieve confluent monolayers. After 30 minutes of inactivation of plasma samples at 56°C, a sequence of seven dilutions (from 1:5 to 1:320) was prepared. The virus aliquot was thawed at room temperature, thoroughly mixed, and diluted to a concentration of 500 pfu/mL in a biohazard safety hood. Equal volumes of plasma and virus dilutions were mixed and incubated for 60 minutes at 37 °C and 5 % CO_2. Then the cells were rinsed with PBS and inoculated with 20 µL of virus-plasma mixture in triplicates. On each plate positive and negative controls were performed in triplicates. After the cells were incubated for 60 minutes at 37 °C and 5 % CO_2 and washed with PBS, 100 µL CMC medium (0.75 % CMC, MEM, 2% FBS, 1x NEAA) was added. The 96-well plates were incubated for three days at 37 °C and 5 % CO_2. The supernatant was discarded, the assays

were coated and dyed with crystal violet (0.1 % crystal violet, 13% formaldehyde) at 4 °C, washed and dried for visual examination (1).

2.2.5.3. NT90-TBEV determination in TBEV-PRNT

The plasma dilution that resulted in a 90% inhibition of virus replication was seen as a neutralizing antibody titre – NT90-TBEV. A particular plasma dilution was only considered to have a confirmed neutralizing effect if both the lower dilution was also neutralizing, and the next higher dilution was no longer neutralizing. The NT90-TBEV was determined by initially calculating the mean plaque count of the positive control and multiplying it by 0.1 to derive the cut-off value representing a 90% plaque reduction. The mean plaque count for each dilution triplicate was assessed and compared against this cut-off value. The initial plasma dilution that resulted in a mean plaque count below this established cut-off value was considered the NT90-TBEV for the plasma sample. During test development, an imprecision of ± one titer level in the titres of the same study participants was observed and considered non-significant (1).

	1	2	3	4	5	6
A	NC	NC	NC	PC	PC	PC
B	Sample 7 day 28 1:20	Sample 7 day 28 1:40	Sample 7 day 28 1:80	Sample 7 day 28 1:160	Sample 7 day 28 1:320	Sample 7 day 28 1:640
C	Sample 7 day 28 1:20	Sample 7 day 28 1:40	Sample 7 day 28 1:80	Sample 7 day 28 1:160	Sample 7 day 28 1:320	Sample 7 day 28 1:640
D	Sample 7 day 28 1:20	Sample 7 day 28 1:40	Sample 7 day 28 1:80	Sample 7 day 28 1:160	Sample 7 day 28 1:320	Sample 7 day 28 1:640

	1	2	3	4	5	6	PC Ø	Cut-off
A	0	0	0	53	57	44	51.33	5.13
B	0	0	0	0	3	4		
C	0	0	0	3	2	5		
D	0	0	0	2	3	8		
Plaques Ø	0	0	0	1.67	2.67	5.67		
Neutralisation	yes	yes	yes	yes	yes	no		

Figure 10. NT90-TBEV determination in TBEV-PRNT.

2.2.6. Indirect Immunofluorescence Tests (IIFT)

For standardized analyses, the titerplane technique (129) and the automated washing system MERGITE! (EUROIMMUN AG, Lübeck, Germany) was used. Regarding the plasma dilutions for the IIFT, the same dilution levels, i.e. 1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640 were chosen, because of the comparability to the dilutions tested in TBEV-PRNT-90. Also higher dilution levels such as 1:1280, 1:2560 and 1:5120 were tested since many titres were described in the literature that were this high or even higher (129). The detection of Ig-G or Ig-M antibodies was performed by separate incubation of diluted samples from subjects to be tested. For Ig-M detection, samples were prepared with a rheumatic factors absorbent (EUROSORB, 2% Tween) in order to remove rheumatic factors and Ig-G from plasma. Biochips were incubated with plasma samples, followed by the addition of Fluorescein isothiocyanate (FITC)-labeled anti-human Ig-M or Ig-G. A washing step using PBS-Tween pH 7.2 (MERGITE!) was conducted after each 30-minute incubation period at room temperature. Slides were coated with glycerol and cover glass before examination under a fluorescent microscope (EUROStar 3 PLUS, EUROIMMUN AG, Lübeck, Germany) at a wavelength of 460-480nm. Antibody titers were determined across serial two-fold dilutions ranging from 1:10 to 1:5120, including appropriate controls. Any discernible perinuclear fluorescence for Ig-M or Ig-G was regarded as a positive reaction. Samples with no antibody titre at 1:10 were considered negative, samples with a titre of 1:5120 were considered as ≥1:5120 (1).

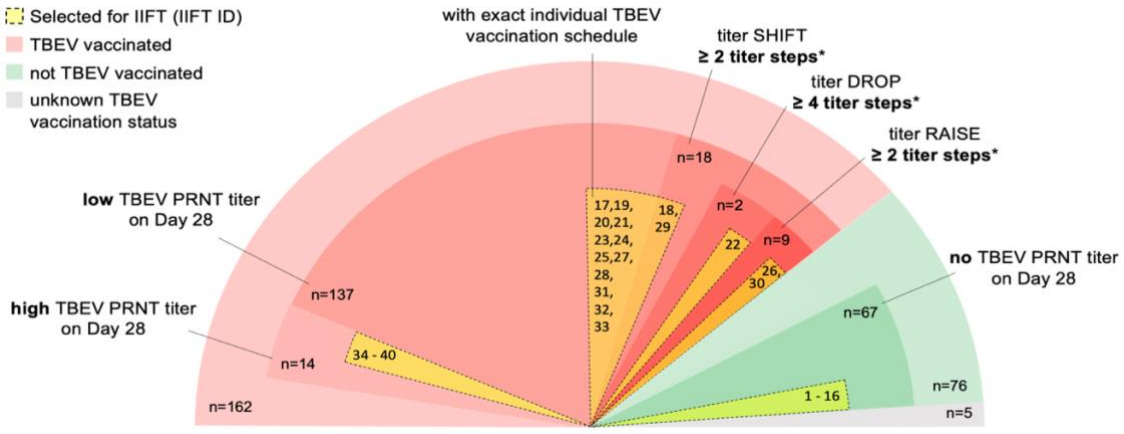

Figure 11. Selection of subjects tested in IIFT.
Selection of 40 subjects tested on flavivirus-specific Ig-M and Ig-G in IIFT after NT-TBEV was determined. Viruses tested: YFV, TBEV, WNV, JEV, DENV 1-4, ZKV; No titre = titre < 1:10, low titre = titre ≥ 1:10 - ≤ 1:160, high titre = ≥ 1:320. *prior to and after YF17D vaccination.

Plasma samples of 40 study participants were tested for Ig-M as well as Ig-G reactive to TBEV, WNV, JEV, YFV, DENV (types I-IV) by using EUROIMMUN Flavivirus Mosaic 3 and ZKV IIFT assays (EUROIMMUN AG, Lübeck, Germany) according to the manufacturer's instructions (Figure 12).

Participants were randomly selected based on their self-reported history of TBEV vaccination and TBEV-PRNT results (Figure 11). N=16 of TBE-naïve (without self-reported history of TBE vaccination and no NT90-TBEV in TBEV-PRNT on day 28) were selected as negative control. Among TBE immunized (with self-reported history of TBE vaccination and NT90-TBEV in TBEV-PRNT at baseline as well as on day 28), care was taken to test an equal number of study participants across all titre levels. Additionally, study participants among TBE immunized were selected, where exact vaccination schedules and administered vaccinations were documented (n=14 out of 16). The exact individual vaccination schedules can be found in appendix (Table 6). Also samples that showed titre shifts ± ≥ 2 from day 0 to day 28 in TBEV-PRNT (n=5) were included. Study participants with discrepancies from NT-TBEV at baseline and day 28 in TBEV-PRNT to self-reported history of TBEV vaccination were excluded from testing.

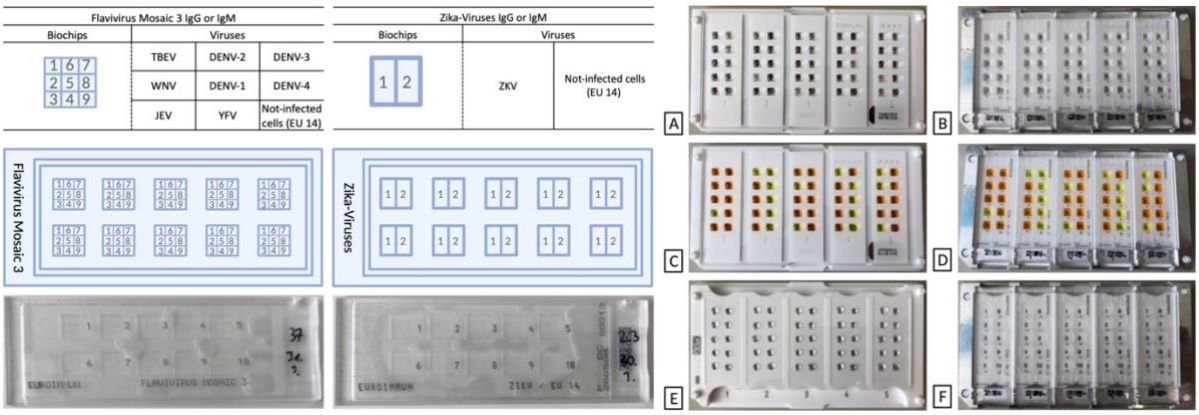

Figure 12. Assignment of biochips of EUROIMMUN plates in use and titerplane technique. The left-hand illustration shows both a schematic sketch and a real-life representation of the structure of the two different test panels used. The individual steps of the Titerplane technique were documented on the right-hand side: A: Application of the plasma samples on the glass plates, B: Incubation of biochips with plasma samples, C: Application of FITC-labelled anti-human Ig-M or Ig-G on glass plates, D: Incubation of biochips with FITC-labelled anti-human Ig-M or Ig-G, E: Application of glycerol on glass plates, F: Sealing of biochips with cover plate.

2.2.7. Fluorescence Reduction Neutralization Test (FluoRNT)

The determination of the neutralizing antibody titers against YF17D (NT-YFV) by a Fluorescence Reduction Neutralization Test (FluoRNT) was performed by doctoral student Lisa Lehmann. The test was performed as described in Santos-Peral *et al.,* (2024) (1).

2.2.7.1. NT-YFV determination in YFV-FluoRNT

The NT-YFV was the maximum serum dilution able to neutralize 80 and 90% of virus infection.

2.2.8. Analyses and Statistics

All data were statistically analyzed by using GraphPad Prism (Version 9). All data were tested for normal distribution and were considered not normally distributed.

Statistical significance between two paired groups was therefore defined using Wilcoxon tests and Spearman correlation with p values <0.05. In the case of unrelated groups, statistical significances were defined with Mann-Whitney tests with p values <0.05.

Definition of box-and-whister-plots: The middle line represents the median of the measured values. The box includes the lower and the upper quartiles (25 – 75%). The whiskers show the minimum and maximum values.

2.2.9. Illustrating Figures and Tables

All Figures were designed using a free version of an online illustrating tool on biorender.com. All tables were created using Microsoft Excel and Microsoft Powerpoint.

3. Results

3.1. Description of study participants

The majority of the study cohort of n=242 were females (n=166; 68.6%) and mean age of men (26.6 years) was slightly higher than of women (25.3 years). Body mass index was in normal range (18.5 – 24.9) for most participants (n=202; 83.5%) with some outliers (n=40; range 17.7-46.4). Most individuals self-reported a history of TBEV vaccination sometime >4 weeks prior study inclusion (n=161; 66.5%).

		All	Self-reported TBE vaccination	Without self-reported history of TBE vaccination	TBE immunized	TBE naïve
TBEV vaccination status			yes	no	yes	no
PRNT TBE Day 0					yes	
PRNT TBE Day 28					yes	no
Total n		242	161	76	138	67
Sex n (%)	f	166 (68.6)	109 (67.7)	54 (71.0)	94 (68.1)	47 (70.1)
	m	76 (31.4)	52 (32.3)	22 (29.0)	44 (31.9)	20 (29.9)
Mean age (range)	f	25.3 (19.0-46.0)	24.8 (19.0-46.0)	26.1 (20.0-40.0)	24.8 (19.0-46.0)	25.6 (20.0-38.0)
	m	26.6 (20.0-47.0)	26.7 (20.0-47.0)	26.5 (21.0-35.0)	26.5 (20.0-47.0)	26.4 (21.0-35.0)
BMI (range)	f	21.9 (17.7-46.4)	22.0 (18.0-46.4)	21.6 (17.6-28.2)	22.1 (18.0-46.4)	21.6 (17.6-27.8)
	m	23.3 (18.1-32.4)	23.7 (18.9-32.4)	22.7 (18.1-26.6)	23.4 (18.9-32.4)	22.6 (18.1-26.6)

Table 1. Characteristics of study cohort regarding sex, age and BMI.
Table includes characteristics of subgroups shown in Figure 15: TBE naïve = without self-reported history of TBE vaccination and no NT90-TBEV in TBEV-PRNT on day 28, TBE immunized = with self-reported history of TBE vaccination and NT90-TBEV in TBEV-PRNT at baseline as well as on day 28. Data from study participants with unknown self-reported history of TBE vaccination not shown.

3.2. NT90-TBEV in TBEV-PRNT

Data can be found in appendix (Table 2, Table 3).

3.2.1. NT90-TBEV on day 28

All n=242 study participants (n=161 of TBE vaccinated, n=76 of TBE non-vaccinated and n=5 with unknown self-reported history of TBE vaccination) on day 28 were tested. N=140 (86.9%) of the TBE vaccinated showed NT90-TBEV, while only 21 (13.0%) did not present detectable NT90-TBEV. Among the TBE non-vaccinated, n=67 (88.1%) did not have a detectable NT90-TBEV, whereas n=9 (11.8%) revealed a NT90-TBEV contrary to their self-reported history of

TBE vaccination. Of the n=5 with unknown vaccination history, n=3 presented no NT90-TBEV and n=2 showed detectable NT90-TBEV. The most frequent NT-TBEV (n=37) was detected at a dilution level of 1:80, 91.9% of which were TBE vaccinated. This was followed by the same proportion (n=29) at dilution levels 1:20 and 1:40, as well as n=23 at 1:10. The remaining 13.6% of NT-TBEV were distributed among the higher dilution levels 1:160 (n=19), 1:320 (n=11) and 1:640 (n=3), with only one of the n=33 with NT90-TBEV higher than 1:160, who was not TBEV vaccinated (Figure 13).

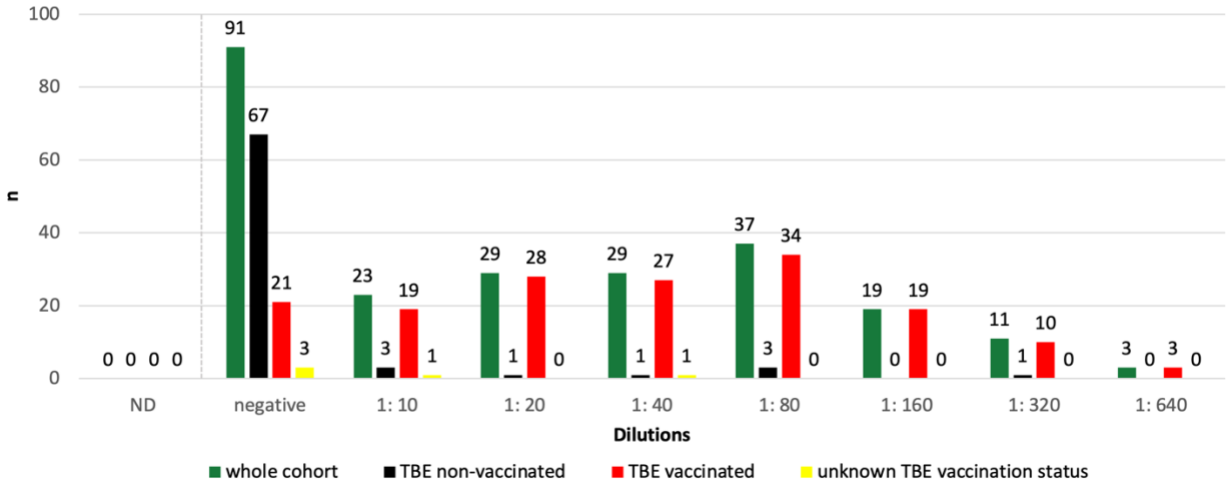

Figure 13. Distribution of NT90-TBEV on day 28.
The n= absolute numbers of NT90-TBEV on day 28 (at dilution levels 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, negative= no NT90-TBEV detectable at a dilution of 1:10) resulting from the TBEV-PRNT of the whole study cohort (green) as well as individually of TBE non-vaccinated (black), TBE vaccinated (red) and study participants with unknown TBE vaccination status (yellow) are shown. ND= not determined.

Potentially modulating factors, such as gender and sex on NT90-TBEV must be considered: Both were tested for statistically significant differences in unpaired t-tests: Sex demonstrated no significant differences in NT90-TBEV in TBE vaccinated (p=0.29). Also no significant differences between age (classified as <25 years, ≥25 years) and NT90-TBEV of all TBE vaccinated (p=0,72) and more differentially of male TBE vaccinated (p=0,29) or female TBE vaccinated (p=0,81) was demonstrated.

3.2.2. NT90-TBEV on day 0

No self-reported history of TBE vaccination combined with no detectable NT90-TBEV on day 28 indicate no NT90-TBEV on day 0, thus no TBEV-PRNT (ND) on day 0 for n=67 subjects were

performed. In addition, for n=2 of the TBE non-vaccinated with NT90-TBEV, TBEV-PRNT on day 28 could not be performed due to insufficient plasma. Ultimately, n=173 samples (n=161 of TBE vaccinated, n= 7 of TBE non-vaccinated and n=5 with unknown self-reported history of TBE vaccination) on day 0 were tested. N=144 (89.4%) of the TBE vaccinated showed NT90-TBEV, with n=17 (10.6%) showing no NT90-TBEV. Among the 7 tested TBE non-vaccinated with NT90-TBEV on day 28, respectively n=6 also showed NT90-TBEV on day 0 and only one had no detectable NT90-TBEV.

The most common findings of NT-TBEV on day 0 were at dilution steps of 1:40 (n=35) and 1:80 (n=34), with n=67 being TBE vaccinated. This was followed by n=34 at a dilution level 1:80, n=25 at 1:20, n=24 at 1:10, as well as n=22 at 1:160. The remaining 4.9% of NT90-TBEV were distributed among the two highest dilution levels 1:320 (n=9) and 1:640 (n=3), with only one of n=12 with NT-TBEV > 1:320, who was not TBEV vaccinated (Figure 14).

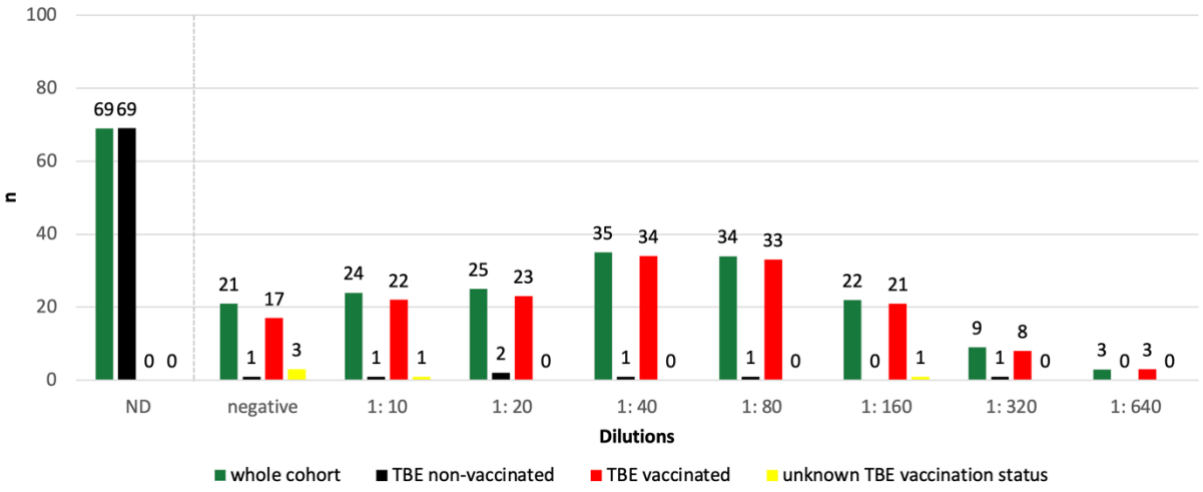

Figure 14. Distribution of NT90-TBEV on day 0.
The n= absolute numbers of NT90-TBEV on day 0 (at dilution levels 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, negative= no NT90-TBEV detectable at a dilution of 1:10) resulting from the TBEV-PRNT of the whole study cohort (green) as well as individually of TBE non-vaccinated (black), TBE vaccinated (red) and study participants with unknown TBE vaccination status (yellow) are shown. ND= not determined.

Showing an overview of the results of TBEV-PRNT (Figure 15), two subgroups emerge in comparison: One group titled TBE naïve represents 67 subjects without self-reported history of TBE vaccination and no detectable NT90-TBEV on day 28 after YF17D vaccination (circled in black). The second group TBE immunized includes 138 subjects with self-reported history

of TBE vaccination and detectable NT90-TBEV on day 28 and day 0 (circled in red). The further analyses compare and describe the two opposing groups.

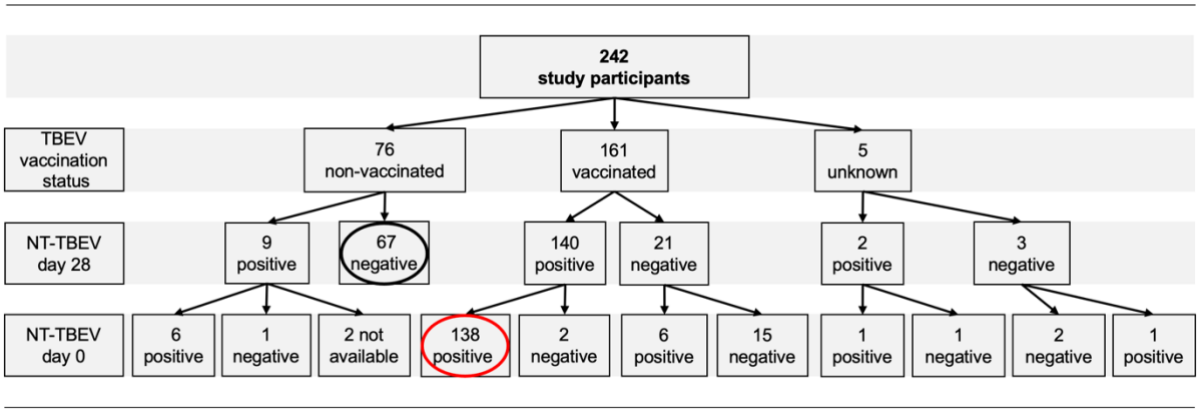

Figure 15. Overview of results of TBEV-PRNT.
NT90-TBEV of all 242 study participants at baseline and day 28. Positive: NT90-TBEV detectable at a titre of ≥ 1:10 in TBEV-PRNT, negative: no NT90-TBEV detectable at a dilution of 1:10 in TBEV-PRNT. Black circle = TBE naïve, Red circle = TBE immunized.

3.2.3. Comparison of NT90-TBEV day 0 and day 28

A total of 173 subjects (71.5%) underwent NT-TBEV determination on both day 0 and day 28. After completion of the TBEV-PRNT, it was observed that a few subjects showed a change in NT90-TBEV from day 0 to day 28 according to the TBEV-PRNT (Figure 16). In n=85 (49%) there was no change and in n=70 (40.5% of NT90-TBEV determined) there was a deviation in NT90-TBEV of one level up or down. In addition, there were differences in the NT90-TBEV of ± 2-4 dilution levels in n=18 subjects (10.4%).

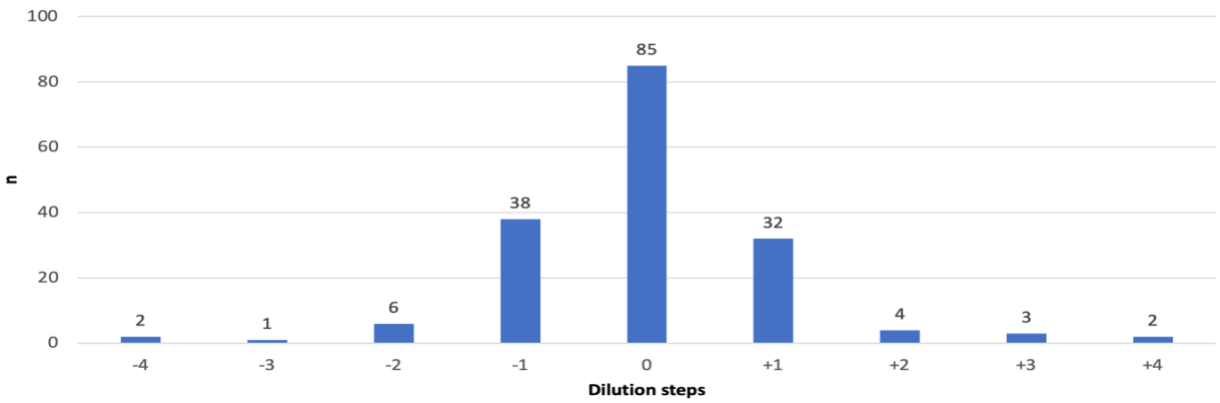

Figure 16. Distribution of changes in NT90-TBEV from day 0 to day 28.
The n= absolute numbers of study participants without a change in NT90-TBEV or with a difference of ± 1 – 4 dilution levels in their NT90-TBEV from day 0 to day 28 are shown.

3.2.4. Repetition of TBEV-PRNT of subjects with differences in NT90-TBEV from day 0 to day 28 of ± 2-4 dilution levels

For n= 15 out of 18 study participants with ± 2-4 dilution levels difference in NT90-TBEV from day 0 to day 28, samples from a different batch were ordered, repeated testing in TBEV-PRNT was absolved and the following was analyzed: At baseline n= 11 out of 15 showed the exact same NT90-TBEV as in the original testing, n= 3 presented a difference of ± 1 titre level and n= 1 showed a difference of 3 titre levels. On day 28, n= 2 showed the same NT90-TBEV as before, n=7 displayed a difference of ± 1 titre level and n=6 demonstrated differences of ± 2-3 dilution steps. By closely looking at the study participants itself only concerning the retesting, n= 7 showed no shift or differences ± 1 titre level from day 0 to day 28. Still n= 8 showed differences of ± 2-5 titre levels.

3.2.5. Influence of YF17D vaccination on TBEV neutralizing activity

When analyzing NT90-TBEV by using TBEV-PRNT and including all n=173 study participants where a measurement prior to (day 0) as well as on day 28 after YF17D vaccination was available, no significant changes in titres could be observed (Figure 17).

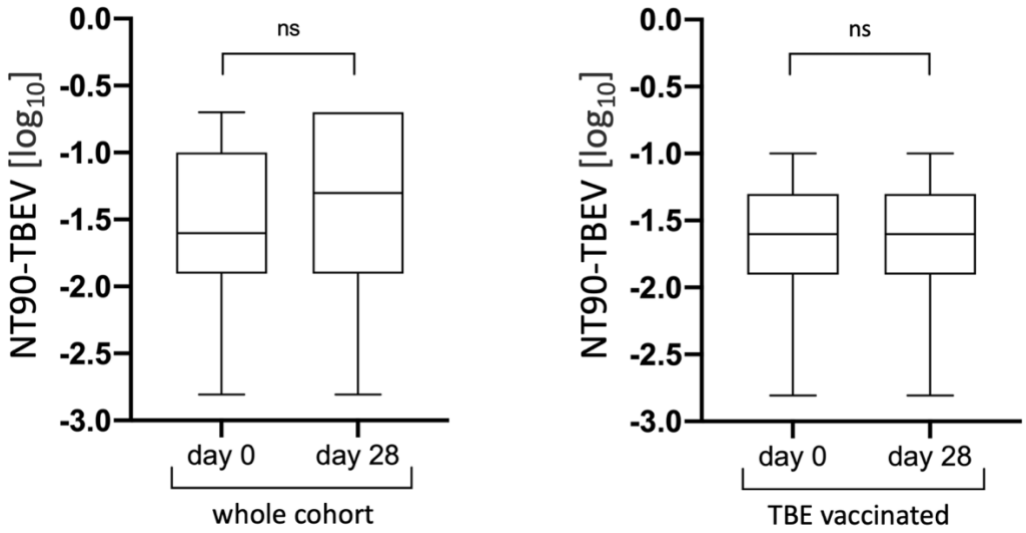

Figure 17. NT90-TBEV titres day 0 and day 28.
Shown as box-and-whisker-plots. For a more detailed illustration, NT90-TBEV are shown as logarithm with a base of 10: $\log_{10}$(dilution level) = *x*. ns= non-significant difference.

3.3. NT-YFV in FluoRNT, determined by Lisa Lehmann

Data can be found in appendix (Table 4).

3.3.1. Influence of pre-existing TBEV immunity on YFV neutralizing activity following YF17D vaccination

The YF17D vaccination induced a significant ($p<0.0001$) NT90-YFV increase in TBE naïve as well as in TBE immunized subjects (day 28 vs. day 14).

When comparing titres of NT90-YFV on day 14 as well as day 28 following YF17D vaccination between n=67 of TBE naïve (with likely no pre-existing TBEV immunity) and n=138 of TBE immunized (with likely pre-existing TBEV immunity), no significant differences between groups could be detected (Figure 18).

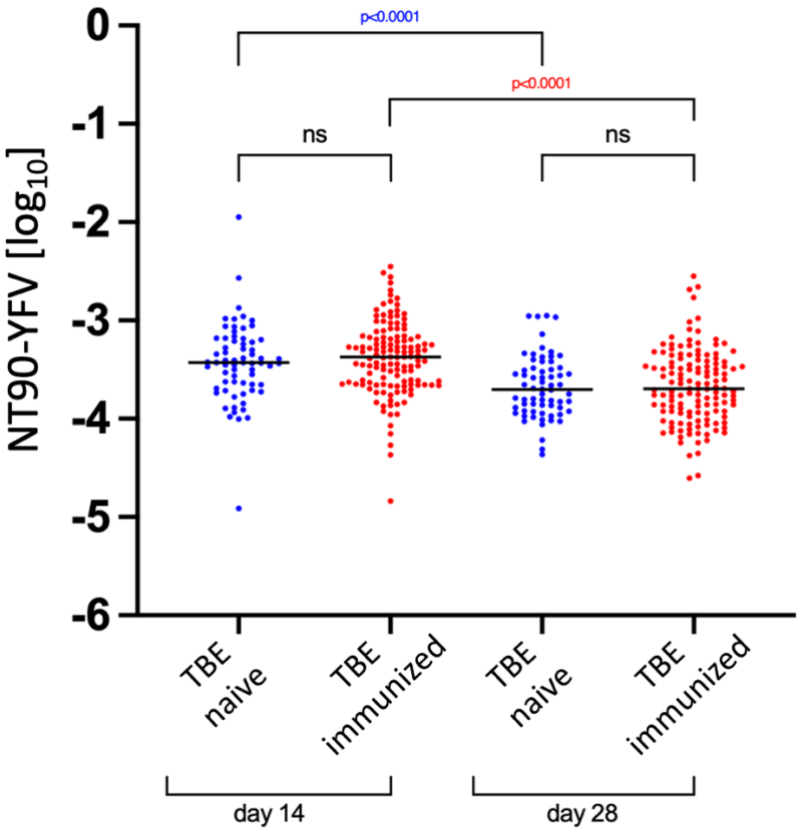

Figure 18. NT90-YFV day 14 and day 28 – TBE naïve vs. TBEV immunized.
ns= non-significant difference.

3.4. Correlation of NT90-TBEV day 0 and day 28 with NT90-YFV day 28 in TBE immunized

No correlation between NT90-TBEV from before (r= 0.1108) as well as on day 28 after (r= 0.1682) and the newly acquired NT90-YFV on day 28 after YF17D vaccination could be found.

There was a significant (p<0.0001) difference between NT90-TBEV day 28 and the newly acquired NT90-YFV day 28 of TBE immunized (Figure 19). Also, for the TBE immunized, the NT90-TBEV at day 0 were significantly (p<0.0001) different to NT90-YFV day 28 (Figure 20).

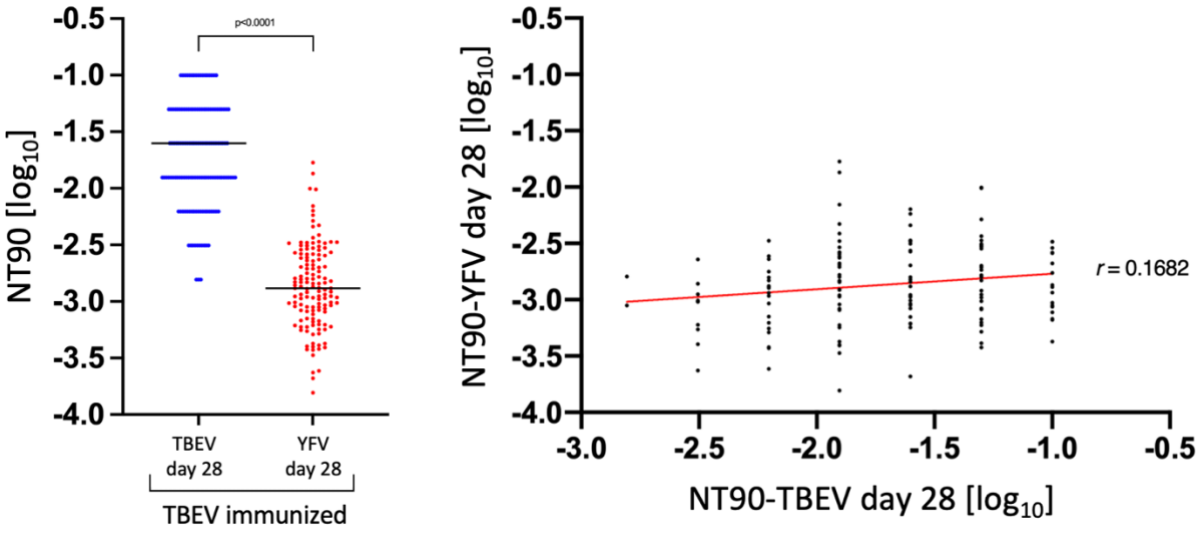

Figure 19. NT90-TBEV day 28 – NT90-YFV day 28: TBEV immunized.

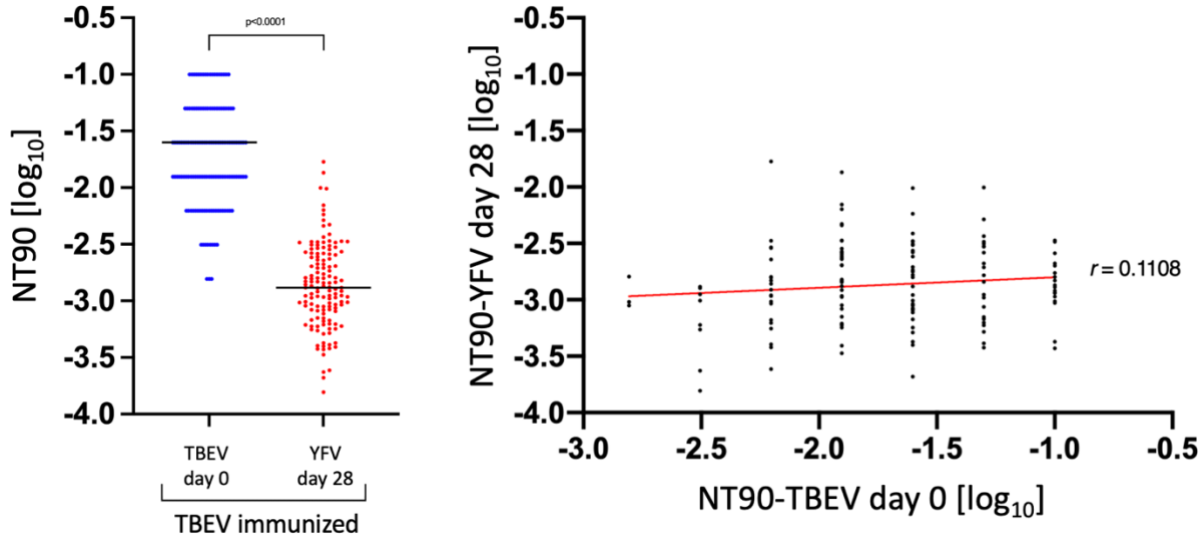

Figure 20. NT90-TBEV day 0 – NT90-YFV day 28: TBEV immunized.

3.5. IIFT

After finishing up TBEV-PRNT, by now using IIFT, Ig-M and Ig-G responses were examined in TBE immunized and TBE naïve for cross-reactivity with other members of the Flaviviridae family. Ig-M and Ig-G binding against DEN1-4, JEV, WNV, YFV, TBE, and ZKV were measured.

With respect to the Flavivirus Mosaic 3 kit, a positive control for Ig-M was only available for DENV and therefore weakly positive for TBEV, YFV, JEV and consistently negative for WNV in all assays (Figure 22).

Data can be found in appendix (Table 5).

3.5.1. Ig-M

At baseline none of the selected study participants, neither from the TBE naïve nor the TBE immunized showed any Ig-M antibodies against any flaviviruses tested. After YFV vaccination, with exceptions almost all of them showed Ig-M against YFV (93.7% of TBE naïve compared to 79.2% of TBE immunized), while no Ig-M was detected in any other flaviviruses (Figure 21).

3.5.2. Ig-G

Whereas only 2 of 16 subjects (12.5%) of TBE naïve presented pre-existing Ig-G antibodies merely against TBEV, among TBE immunized Ig-G antibodies were largely contributed throughout all tested viruses at day 0 (100% TBEV, 66.7% WNV, 75.0% JEV, 66.7% YFV, 66.7% DENV1, 75.0% DENV2, 70.8% DEN3, 62.5% DENV4 and 66.7% Zika).

After the YFV vaccination all TBE immunized showed Ig-G titres throughout all viruses tested (98.1% Ig-G titres ≥7), independent of non/pre-existing Ig-G at baseline. All of TBE-naïve showed Ig-G against YFV at day 28 post YFV vaccination, however, two exceptions of TBE naïve who showed low Ig-G titres against TBEV at baseline. One of them also showed high Ig-G titres against all flaviviruses (Figure 21).

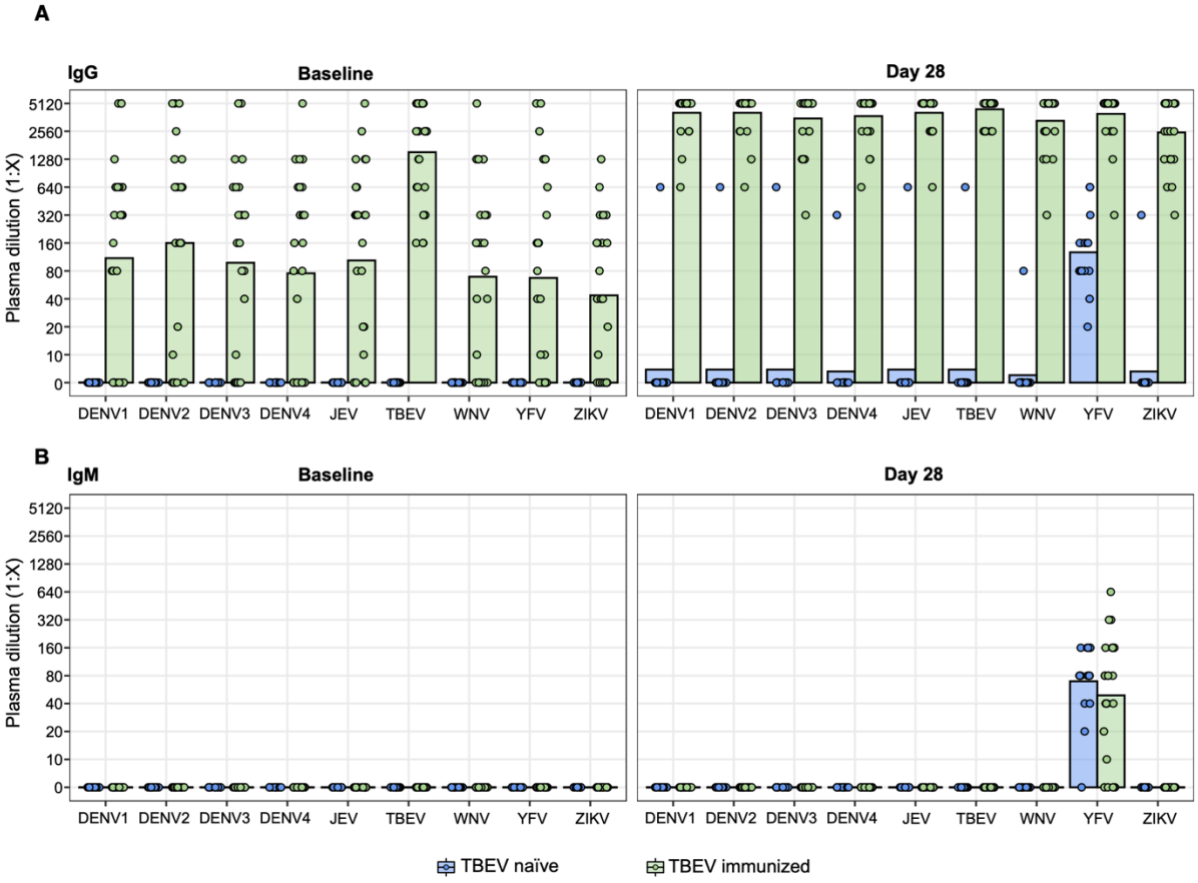

Figure 21. Ig-M and Ig-G cross-reactivity before and after YF17D in TBE immunized and naïve.

(A) showing Ig-G and (B) Ig-M cross-reactivity in selected TBE naïve (blue) and TBE immunized (green) study participants before and 28 days after YF17D vaccination, detected by IIFT for DENV1-4, JEV, TBEV, WNV, YFV and ZKV (with kind permission by Santos-Peral *et al.,* (2024), submitted for publication (1)).

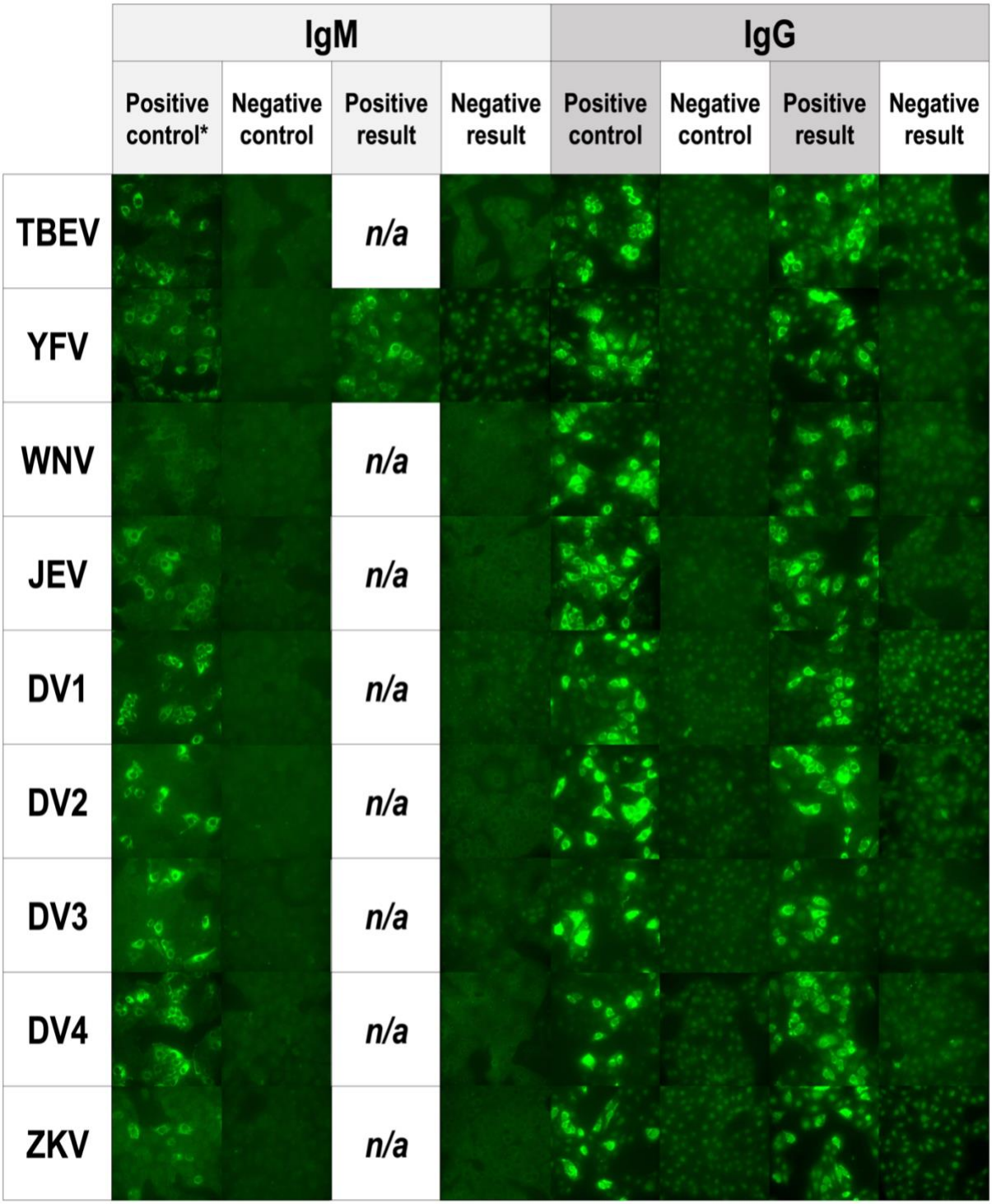

Figure 22. Exemplary immunofluorescence microscopy images of flavivirus-infected EU14 cells.
n/a: no positive images/results were observed.

4. Discussion

4.1. Summary

As more and more flaviviruses spread on a global scale, thus increasing the risk of multiple infections with different flaviviruses, it is of paramount importance to gain a deeper insight and acquire a comprehensive understanding of existing immunities against these viruses. Since the development of the live-attenuated vaccine YF17D, the vaccine against yellow fever is one of the most important tools in dealing with viruses from the flavivirus family. Although some studies examined this (132–134), the underlying immunological mechanisms of action of the extremely potent vaccine against yellow fever are still beyond current knowledge. This doctoral thesis contributes to investigating the influence of NT-TBEV on the immune response after YF17D vaccination. By determining the neutralizing TBEV antibodies at baseline and 28 days after YF17D vaccination, this doctoral thesis shows whether NT-TBEV differ or remain unchanged by YF17D vaccination. In addition, by including NT-YFV formed after vaccination, correlations of the immune response to YF17D vaccination with NT-TBEV could be observed. On the other hand, IIFTs were used to inspect the development of cross-reactive antibodies from eight different flavivirus infections in flavivirus-naïve versus TBE vaccinated study participants.

To correlate NT-TBEV with the immune response to YF17D vaccination, a longitudinal cohort of 242 individuals who were either TBE vaccinated or flavivirus naïve was established. Even though NT90-TBEV were found to be heterogeneously distributed across all titre levels, no significant alteration of NT90-TBEV was observed in TBE immunized. This indicated no influence of the yellow fever vaccine to previous TBE immunisation. Conversely, the NT-YFVs induced by YF17D vaccination in TBE naïve and TBE immunized individuals behave in the same way, which means that TBEV pre-immunity had no impact on the formation of NT-YFVs. To also demonstrate possible cross-reactive antibodies in TBE naïve and TBE immunized individuals, Ig-M and Ig-G responses to the most prominent flaviviruses were measured using IIFT: TBEV, YFV, WNV, JEV, DENV 1-4, ZKV. After vaccination with YF17D, TBE naïve as well as TBE immunized showed an expected increase in Ig-M antibodies specifically against YFV, like other studies showed (129). In contrast, the Ig-G responses showed an interesting contrast

between TBE naïve and TBE immunized: In study participants who were TBE vaccinated, there was already cross-reactivity across all flaviviruses due to the TBE vaccination prior to the YF17D vaccination. This picture of cross-reactivity was clearly reinforced by the yellow fever vaccination. In contrast, the Ig-G response in TBE naïve to YF17D vaccination was almost exclusively directed against YFV, underlining the property of the YF17D vaccine in TBE naïve to prevent the formation of cross-reactive antibodies.

4.2. Methods

4.2.1. Cohort and study setup

The study participants were recruited on a voluntary basis in a prospective longitudinal study. At the beginning, the participants filled out a questionnaire which, together with the presentation of the vaccination cards, was to determine whether any natural flaviviral infections occurred or vaccinations against YFV, TBEV and JEV were already carried out more than 4 weeks before the study. The new dengue vaccination, which is available in Germany since February 2023 (135), does not play a role in our study, because only participants were included from 2015 - 2019. Regarding their vaccination history, 5 study participants answered "unknown" to the question about previous TBEV vaccination, and one study participant reported already being vaccinated against JEV. However, since the vaccination certificates were used to strengthen the leading criteria of self-reported history of TBE vaccination, only the participant who was vaccinated against JEV was excluded. The participants who gave an unknown vaccination history were nevertheless tested in the TBEV-PRNT out of interest.

Furthermore, it should be noted that due to the choice of the study model, it was not possible to randomise the study participants, which may lead to bias: most of the study participants were female, and the mean age of all participants was quite young (Table 1), which didn’t represent the regional population (136). In addition, the clear majority was vaccinated against TBEV. However, this was plausible due to the official TBE vaccination recommendation in the region where the study took place (137). Unfortunately, the exact vaccination schedules and administered vaccines of the TBEV vaccinations at study inclusion were not completely documented (only for n=16). Based on which, additional information regarding possible differences in vaccination titres due to different vaccines or time intervals could have been

obtained observed more precisely. Compared to other studies that conducted clinically randomized trials and thus obtained more balanced study populations (120), this model had its limitations due to its longitudinal character. Still, compared to others (107,115,120), with 242 participants this study obtained a large cohort, to offer a relatively deeper insight into a distinct pattern of a confection with two of the main flaviviruses.

4.2.2. Plaque Reduction Neutralization Test for TBEV

The neutralizing activity plays a central role in humoral immunology and diagnostics. It is discussed that cellular infections as well as viral replication are reduced (130). The PRNT is considered the gold standard in the detection of neutralizing antibodies targeting YFV or TBEV (138,139). However, since there are different protocols for this test, a protocol based on Litzba *et al.,* (2014) was developed and was considered the most suitable (124). A review by Demicheli *et al.*, (2009) described a seroconversion after TBE vaccination given by a positive ELISA or a neutralizing antibody titre of ≥1:10 in a neutralization test (140). In accordance with the WHO, these neutralizing antibodies serve as correlate of protection and neutralizing antibody titres of ≥1:10 function as seroprotective (141,142). To deliver results that are as comparable as possible and therefore strengthen this method, the described antibody titres, which serve as ≥1:10 to be seroprotective, were also adhered to.

With a large cohort of 242 participants, the titre determination with the TBEV-PRNT at two different times (Figure 7) and a test duration of 4-5 days, a large turnover was required. This necessitated sufficient care in cell culture as well as in any other laboratory activity. In addition, enough plasma should be available, which although there were shortages with two samples, and no determination of NT-TBEV was possible in this case. In order to save resources and as it was assumed that TBE non-vaccinated without NT90-TBEV on day 28 would not have a NT-TBEV on day 0, they were not tested for their NT-TBEV at day 0. Accordingly, false negatives might be hidden here. These doubts could be removed in future studies with more detailed testing. Concerning the turnover, unlike others, no antibiotics were used in cell culture to prevent bacterial contamination (143). At one hand fully contaminated plates couldn’t be used and slowed down the turnover of tests and partially contaminated plates made it more difficult to visually examine still uncontaminated wells on

the same plate. At the other hand to minimize contaminations, we conducted the TBEV-PRNT under biosafety level 3** at the Bundeswehr Institute for Microbiology in Munich, Germany. This entailed the BSL2 standards and additionally a dedicated laboratory including a microbiological safety cabinet, with access granted to selected scientists who must wear special protective equipment. Moreover, in the case of a large turnover, it must be taken into consideration that, for example, sample mix-ups or incorrectly long or short incubation times could have occurred during the test procedure. Pre-analytical mistakes could also have occurred during blood collection or aliquoting. However, in case of doubt, aliquots from another batch could be provided and the test repeated to minimise this source of error. For each dilution level of each study participant tested, three wells on each plate were incubated to calculate more meaningful cut-off values (Figure 10), thus to be able to determine more reliable titres and to avoid the sources of error mentioned above. Due to imprecision of our TBEV-PRNT the NT-TBEV could differ in ± one titer level in repeated testing and were considered a non-significant titre shift. Likewise concerning the manual plaque counting by visual examination, it must be taken into account that its non-automized visual counting were subjective and depended on the analyst's variability. In contrast, automated counting methods are a less error-prone alternative (108). For the reasons given above, similar assays, such as FRNT, are already being developed to provide faster and more objective results by automized processes (144,145).

4.2.3. Indirect Immunofluorescence Test

In addition to the determination of NT-TBEV, this doctoral thesis also addressed the detection of possible cross-reactive antibodies, which, as in the case of the previously described ADE, can lead to a misdirected immune response after contact with flaviviruses: For this purpose, Ig-M and Ig-G antibody titres were measured with the help of the IIFT. Ig-M are relatively quickly secreted antibodies which try to fight the pathogen in about 3-5 days after contact with it and reduce after about 1-2 months. Now Ig-G are formed, which should act more specifically and quickly in the case of further infections (Figure 5).

Those tested in the IIFT were selected on the basis of the test results from the previous TBEV-PRNT, as we were particularly interested in the IIFT signatures of the TBE naïve versus the TBE

immunized. Since, as described above, the exact vaccination regimens of very few TBE immunized were documented at study inclusion, this information was included alongside a general range of different titre constellations. But since very few of those selected were not vaccinated with only one of the two vaccines (Table 6), this approach was abandoned and we focused on the differences of the TBE naïve versus the TBE immunized. With regard to the titre levels tested, we were guided on the one hand by other studies (129) and on the other hand by the dilution levels we already used in the TBEV-PRNT, in order to establish the greatest possible similarities in comparison. Since our test approach was limited to the maximum dilution of up to 1:5120 due to the many dilutions tested, we were unfortunately unable to apply higher dilution levels and thus titrate out exact titre levels.

Generally, certain error sources need to be inspected: Firstly, it must be taken into account that the positive control available for Ig-M only for DENV, apart from strong positive signals for YFV, was only weakly positive for TBEV, YFV, JEV and consistently negative for WNV. This was not a problem, as the study participants tested in the IIFT did not show any positive Ig-M results for the remaining viruses, again with the exception of detectable signals for YFV. Additionally, relatively small sample volumes were used and it ran the risk of using insufficiently informative material if the samples were not well prepared. Therefore, aspiration was done carefully and sufficiently often to minimise pipetting errors. In addition, in comparison to others the titerplane technique as well as the MERGITE! Washing automat recommended by EUROIMMUN to reduce manual mistakes were used (129,146). After absolving the test, visual examination was carried out: Even though any specific perinuclear fluorescence was seen as positive, cellular or membrane fluorescence could be misleading. At the beginning of the analyses, however, the visual evaluation was learned under professional guidance by Dr. Dobler as well as double-checked. Nevertheless, it is very dependent on who reads the plates, as there are differences between the readers. But since the plates were tested and visually evaluated by only one analyst as well as typical fluorescence was always compared to positive controls, we tried to counteract this variance. As shown in Figure 5, automated methods such as ELISA are available for testing. As conducted in other studies (108,115,120), this method offers an objective measurement of antibodies and is therefore a reasonable alternative to IIFT and should be considered in study planning.

4.3. Observation: No alteration of TBE pre-immunity

As 173 subjects of our study cohort were tested in TBEV-PRNT, NT90-TBEV were found to be heterogeneously distributed across all titre levels due to the lack of information about exact time points and quantity of TBE vaccination (Figure 13 and Figure 14). 89.5% of them showed 0 or ± 1 titre dilution difference from baseline to day 28. This resulted in a non-significant alteration of NT-TBEV due to a YF17D vaccination (Figure 17). Still 10.4% showed a difference in the NT90-TBEV of ± 2-4 titre dilutions, which may be justified by measurement errors, as described earlier. However, this did not contribute to any significant difference in relation to the cohort as a whole. The presented results were consistent with a study published by Bradt *et al*., (2019) with a very similar study-setup, in which YFV pre-vaccinated and flavivirus naïve patients were administered a TBE vaccine. Similarities can be found as pre-existing Nabs against YFV also did not differ from baseline to day 28 after TBE vaccination. It needs to be stated that their study cohort was more than three times smaller than ours, which demonstrates the comparatively more conclusiveness of our study-setup (115). Additionally, gender and age demonstrated no significant influence on NT90-TBEV at day 28. It should be noted, as described above, that we had more female than male study participants and a very young majority of the study population (Table 1). As already noted, the time interval between TBE vaccinations and our study also may play a role as a modulating factor. However, as these were not documented, it was unfortunately not possible to make any statements about possible influences on NT-TBEV or correlations between the last TBE vaccination dose administered and NT-TBEV.

When looking closely at NT-TBEV analyzed by performing TBEV-PRNT, the 90% cut-off neutralization, as mentioned above, was used. After analyzing the data, we examined very few discrepant titres, meaning either TBE vaccinated without NT90-TBEV at baseline and/or day 28 or TBE non-vaccinated with detectable NT90-TBEV at baseline and/or day 28 (Figure 15). To get an impression for the rough dynamics of the neutralization titres as shown in Figure 10, one should include the observation of the neutralization capacity of the lower as well as of the next higher dilution. The neutralization can result in one higher/lower titre and was considered a non-significant titre shift due to imprecision. A titre shift ± ≥ 2 dilution steps can be recognized, but poorly explained. This could be discussed as analytical uncertainty

either due to pipetting errors or an error in counting the plaques. To further investigate dynamics of neutralization, the plasma dilution was also calculated resulting in an 80% virus neutralization (NT80-TBEV, shown in Appendix Table 2). There can be seen that in quite some of the incongruent NT90-TBEV the 80% neutralization capacity, for example, persists at titre levels ≥ 1:10, while the 90% neutralization capacity may indicate no neutralization ≥ 1:10, meaning there was no NT90-TBEV detectable. This must be taken into account, when having a closer look at discrepant titres:

N=9 subjects of TBE non-vaccinated with a NT90-TBEV on day 28 aroused our interest and, therefore, it was decided to test their NT90-TBEV at baseline (as we did not test NT90-TBEV on day 0 of TBE non-vaccinated without NT90-TBEV on day 28). In two cases, however, the plasma volumes were not enough for further testing. Concerning the remaining n=7: In n=6 also NT90-TBEV on day 0 was found, indicating that in 7.9% of TBE non-vaccinated, in contrary to their self-reported history of TBE, YFV and JEV vaccination as well as history of natural infections, NT90-TBEV could be detected at baseline and day 28. This may be explained by a previous unknown natural infection with TBE, not being recognized as such, since about 70 - 98% of infections with TBE are asymptomatic and infection-related seroprevalence is high in south-west Germany (147,148). By examining the exact titres 66.7% (n=4 out of 6) of TBE non-vaccinated with detectable NT90-TBEV showed titres ≥ 1:160. This was complementing a study by Remoli *et al.*, (2015) where higher NT-TBEV could be observed after natural infection than in TBE vaccinated subjects (149). In the n=1, no NT90-TBEV was detected on day 0 according to the self-reported history of TBE vaccination despite a NT90-TBEV on day 28.

When looking at TBE vaccinated with NT90-TBEV on day 28, as already mentioned, 98.6% (n=138) showed a NT90-TBEV at baseline. Surprisingly, n=2 did not show a NT90-TBEV on day 0. When looking closely at those participants' NT90-TBEV on day 28, both showed a NT90-TBEV at a dilution level of 1:10. Considering titre shifts ± 1 dilution steps as non-significant imprecision, this could be discussed as such. Moreover NT80-TBEV showed one of them with congruent titres at baseline and day 28. As already mentioned, n=21 of TBE vaccinated without NT90-TBEV on day 28 for their NT90-TBEV on day 0 were also tested. Here, n=15 presented also no NT90-TBEV on day 0. In general, it could be observed that vaccinations may not result in a complete or prospective immune protection in 100% of the vaccinated

individuals. Vaccinated individuals may exhibit low or no response to a vaccination, meaning they may not develop sufficient neutralization titres, and are consequently referred to as low or non-responders (150). This could serve as an explanation for these partial results. Furthermore, due to the lack of information about the exact vaccination schedules of each TBE vaccinated with possible overdue booster vaccinations resulting in insufficient neutralization titres, this may be a cause for non-neutralizing or very low neutralizing antibody titres in contrast to their self-reported history of TBE vaccination (151). Continuing looking at n=21 of TBE vaccinated without NT90-TBEV on day 28, discrepancies from day 28 to day 0 were noticeable again: N=6 subjects had a detectable NT90-TBEV on day 0 despite no NT90-TBEV on day 28. Again, by looking at the dynamics of neutralization at each dilution step tested as well as NT80-TBEV, it can be observed that n=5 revealed almost congruent NT80-TBEV at baseline to day 28. This could be explained by non-significant measurement differences, meaning either the titres from day 0 or, conversely, from day 28 showed this uncertainty. When taking NT80-TBEV into account, it can be considered that the neutralization capacity varied from 80% to 90% without hitting the cut-off to show as NT90-TBEV.

N=5 of the 242 subjects (2.1%) could not provide information about their TBEV vaccination history. For this reason, we excluded them from analyzing but not from testing: N=1 presented NT90-TBEV at baseline and day 28, n=2 showed neither NT90-TBEV at baseline nor on day 28. Also two discrepant results appeared, which can also be confirmed by NT80-TBEV.

A closer look at the study participants whose titres exhibit a difference of ± 2-4 titre levels from day 0 to day 28 (see 3.2.4.): The retesting of these subjects showed interesting results: On the one hand, it could be shown that the number of subjects with differences in NT90-TBEV of ± 2-4 titre levels decreases from day 0 to day 28. On the other hand, it can be observed that the NT90-TBEV to be compared differ more from the first test on day 28 than on day 0. Both could be explained by the fact that the TBEV-PRNT started on day 28. Secondly, the quality of the individual plates and thus the individual visual count of this non-automated test also varied from day to day as well as from plate to plate. Nevertheless, this source of error was countered by the fact that only one person conducted and evaluated the tests.

Rounding up the results, 138 of 161 TBE vaccinated subjects presented neutralizing antibody titres at baseline as well as on day 28, which demonstrated a ratio of 85.7% of immunization. When comparing this to the claims of the two licensed TBE vaccines available (FSME-IMMUN® and Encepur®): They claimed a seroconversion in 92-100% of vaccinated persons (152–155), a review by Demicheli *et al.,* (2009) also described a seroconversion of about 87% in 6,586 adults and 1,598 children within 11 randomised clinical trials (140). This means that in the presented study the ratios were slightly undercut. But it is important to note that it is not advisable to easily compare ratios of seroconversion, as no information on how many and which of the two available vaccines were vaccinated, were collected, whether the commercial or rapid vaccination scheme was carried out, whether booster vaccinations were given and, in general, how long ago the last vaccination was given. Accordingly, the NT90-TBEV were also distributed quite heterogeneously. This information was collected for only a few subjects, which were therefore also included in the IIFT. However, since most of the subjects were vaccinated or had booster vaccines with both vaccine manufacturers alternately, it was refrained from further observations and analyses because the information provided was too marginal and diverse. Care was only taken at NT90-TBEV and NT-YFV up to day 28 after YFV vaccination; possible changes after a longer period were not investigated further. Normally, neutralizing Ig-M reach their peak phase after the viraemic phase (day 5-7) until the end of the second week after injection with YF17D and seroconvert to neutralizing Ig-G in the course of the 4th-6th week. NT-YFV could therefore presumably be even higher during the course of the disease than were measured (52). Accordingly, also longer-term measurements of neutralizing antibodies should be considered in future approaches (107).

4.4. No influence of pre-existing TBE immunity on YFV neutralizing activity following YF17D vaccination

Vector-borne diseases are considered to be on the rise as a result of global warming, as predicted by the WHO (122). For people who both originate from TBE endemic areas and travel in Europe and Asia, the risk of infection with both viruses is not unusual. The NT-YFV were measured by Lisa Lehmann (1) and determined for days 14 and 28 after the YF17D vaccination. In both groups, TBE naïve and TBE immunized, there were significant increases in NT90-YFV (Figure 18), which allowed the confirmation of the excellent effect of the

vaccination (52). There were no significant differences in NT90-YFV between TBE naïve and TBE immunized on either day. A closer look at the group of TBE immunized with respect to NT90-TBEV from both day 0 and 28 revealed significant differences to the newly established NT90-YFV from day 28 (Figure 19 and Figure 20). Furthermore, a correlation of NT90-TBEV of day 0 and 28 with the NT90-YFV of day 28 could not be shown, which means that notwithstanding how high the NT90-TBEV seemed to be on any given day, this had no influence on the level of NT90-YFV on day 28. On the one hand, this could of course be related to the timeliness of the respective vaccination: The dates of the last TBE vaccinations administered to the TBE immunized were not known and thus the time intervals here could range from a minimum of 4 weeks to more than 5 or even 10 years. Since booster vaccination is required every 5 years to maintain immunity against TBE, unlike the one-time YFV vaccination, this could be a reason for comparatively lower titres. On the other hand, varying levels of antibody titres above the 90% cut-off may have no decisive effect on their neutralizing capacity. Here, it's interesting to take a look at the study published by Bradt *et al*., (2019): In contrast to the presented study, it was observed that in YFV pre-vaccinated the formation of new antibodies against TBE after vaccination was impaired insofar as there was lower neutralization capacity. This led to the assumption that the YF17D vaccine administered in this study, regarding neutralizing antibodies, circumvented the immunological phenomena described earlier, which culminate in ADE via cross-reactivity, and triggered an immune response that is unchanged compared to TBE naïve individuals (115). In accordance with the cited study, pre-immunity to yellow fever, for example, also showed no modulation of the clinical severity of subsequent dengue disease (156).

Overall, this is suggesting that TBE pre-immunity did not appear to influence the establishment of sufficient NT-YFV. The ability to produce robust neutralization titres, while preventing cross-reactivity in flavivirus naïve individuals, was not observed in other flaviviruses, whether vaccinated or infected (157–159).

4.5. Enhanced panflaviviral cross-reactivity in TBE immunized besides absence of cross-reactivity in TBE naïve individuals after YF17D vaccination

Using IIFTs, a panflaviviral cross-reactivity Ig-G signature in TBE immunized individuals was revealed, which broadened and increased 28 days after YF17D vaccination. It should be noted that the detection of Ig-G and Ig-M against all tested flaviviruses was not performed qualitatively, but semi-quantitatively, and therefore no statements about the neutralization capacity of these antibodies can be made.

By performing IIFT before and after the YF17D vaccination to detect antibodies formed against nine different flaviviruses, TBE naïve as well as TBE immunized subjects showed an expected increase in Ig-M antibodies specifically against YFV, such as other studies showed (129). By studying Ig-G at baseline, two interesting pictures were elaborated: Whereas no Ig-G was detected throughout the TBE naïve at all, TBE immunized already showed cross-reactive activity throughout all flaviviruses tested. After YF17D vaccination, TBE naïve showed an exclusive increase of Ig-G against YFV. Among all TBE immunized, an impressive picture of highly boosted, panflaviviral Ig-G was observed. Due to the selection criterion of the exact vaccination regimen, which were discarded for possible analysis, this information nevertheless confirmed and strengthened the self-reported history of TBE vaccination of the tested study participants in relation to NT-TBEV. With a larger number of TBE immunized of both available vaccines (FSME-IMMUN® and Encepur®) in comparison including detailed protocols and different vaccination regimens (conventional or rapid), interesting observations and analyses would be possible. As the Ig-G tests for TBE immunized on day 28 showed very high titres (≥ 1.5120) across all viruses (only titrated up to the dilution level 1:5120), exact titre values above this were unfortunately missed. To make this possible, it would be advisable for further trials, to titrate further to provide more detailed results.

The findings reported here are in accordance with observations that found cross-reactive antibodies after TBE vaccination (22) and, furthermore, it is in line with the results of another study with JEV-pre-vaccinated patients and subsequent YFV vaccination, in which enhanced cross-reactivities were detected (120). These findings match the results of a study that also investigated the behaviour of antibodies in YFV-pre-vaccinated patients and subsequent TBE

vaccination and also detected an significantly increased cross-reactivity (107). Again, the previously published results were conducted with significantly smaller cohort sizes than in this study, except for those in Chan *et al.,* (2016) (120).

For the discussion of possible mechanisms behind the presented findings, immunological phenomena such as cross-reactivity to ADE must be examined: As the development of a DENV vaccine was the subject of intense research and debate around a dengue vaccine due to ADE, Ripoll *et al.*, (2019) presented high cross-reactive Abs as a correlate to increased ADE (121). Also passive versus active immunization with intravenous TBEV Ig-G concentrates was blamed for ADE (81). Although this could not be confirmed, further trials for passive immunization systems in Europe were discontinued (160,161). It was also shown that ADE could increase the immunogenicity of the YF17D vaccine through cross-reactive, non-neutralizing antibodies (120). Even protective effects of YF17D immunization after prior contact with DENV, JEV and YFV were described (162,163). Also, Schuller *et al.,* (2008) presented results showing that pre-existing TBEV vaccine protection increased Nabs against JEV after the first vaccination with an inactivated JE vaccine candidate. However, this was probably due to cross-neutralizing Abs against TBEV rather than newly formed Nabs against JEV (108). Since in this study it was only tested for the presence of cross-reactive antibodies, but not specifically for ADE, no further statements can be made in this regard. Only in combination with the tests performed on Nabs (TBEV-PRNT and FluoRNT), as well as in accordance to other trials, it could be confirmed in the presented study that YF17D induced neutralizing but non- or very low crossreactive antibodies in flavivirus naïve (25,111). Cross-reactivities, which were present in subjects with TBE vaccine-induced immunity, were enhanced without affecting the neutralizing capacity against either TBEV or YFV.

Overall, this is suggesting that YF17D induced non-crossreactive antibodies in flavivirus naïve subjects, while enhancing cross-reactivities were present in TBE immunized due to TBE vaccination.

5. Conclusion and Outlook

Due to global warming as well as globalization, the broad understanding of immune responses to flaviviruses will draw even more attention of infectiologists as well as laboratory physicians in the near future. Better understanding the effects of pre-existing immunity on the immune responses following vaccination is of great importance for translational infection research: To measure neutralizing antibodies against TBEV, the PRNT remains the methodological tool of choice. In the presented study NT-TBEV before and 28 days after YFV vaccination were analyzed with reference to NT-YFV, measured by use of FluoRNT. Also IIFT was used to observe whether cross-reacting antibodies against other flaviviruses were present due to the pre-existing TBEV vaccination and how these would behave in comparison to TBE naïve individuals after YFV vaccination.

It could be shown that an administered YFV vaccine did not affect already existing NT-TBEV. Similarly, YFV vaccination induced equally strong NT-YFV in both TBE immunized and TBE naïve individuals. Furthermore, enhanced panflaviviral cross-reactivity in TBE immunized subjects as well as the absence of cross-reactivity in TBE naïve individuals after YF17D vaccination could be demonstrated. The need became apparent to investigate even further specific neutralizing and/or cross-reactive epitopes. The presented work is part of a previously published explanatory approach (1), which showed that vaccinations against TBE and subsequently against YFV do not hinder each other's robust immune response, but rather can enhance immunogenicity due to ADE. The data suggested that YF17D induced non-crossreactive antibodies in flavivirus naïve subjects, while enhancing cross-reactivities were present in TBE-immunized due to TBE vaccination.

Long-term effects of YF17D vaccination on NT-TBEV and the long-term influence of pre-existing immunity against TBEV on NT-YFV were not investigated. Similarly, no conclusions could be drawn about the correlation of NT-TBEV with different TBEV vaccinations, vaccination schedules or time interval since the last vaccination. It would be advisable to document accurate vaccination data in future studies. In addition, a more representative study-cohort of the regional or even national population should be proposed in order to further strengthen the clinical significance.

Appendix

5.1. Data of TBEV-PRNT results

A	B	C	D	E	F
1	yes	2	1	1	1
2	no	ND	ND	0	0
3	no	ND	ND	0	0
4	yes	2	1	3	2
5	yes	6	5	7	7
6	yes	3	3	3	3
7	yes	5	5	7	6
8	yes	7	6	7	6
9	yes	5	4	6	5
10	yes	4	4	3	3
11	yes	4	4	4	4
12	yes	4	3	5	3
13	no	ND	ND	0	0
14	yes	6	6	7	6
15	yes	0	0	0	0
16	yes	3	2	2	1
17	yes	5	4	4	4
18	yes	4	3	4	4
19	no	ND	ND	0	0
20	yes	4	4	4	3
21	yes	5	5	6	5
22	yes	4	3	3	0
23	yes	5	4	3	3
25	yes	1	1	5	4
26	yes	0	0	0	0
27	no	ND	ND	0	0
28	yes	7	7	7	7
29	yes	3	2	3	2
30	yes	5	4	4	4
31	no	ND	ND	0	0
32	no	4	4	5	4
33	yes	3	3	2	2
34	no	2	2	2	1
35	yes	2	1	0	0
36	yes	6	5	6	5
37	yes	2	2	2	2
38	yes	0	0	0	0
39	no	ND	ND	0	0
40	yes	5	4	5	4
41	no	ND	ND	0	0
42	yes	3	3	5	4
43	no	ND	ND	0	0
44	yes	3	3	5	5
45	yes	5	4	6	3
46	yes	5	4	5	5
47	yes	3	3	3	3
48	yes	2	2	4	3
49	no	ND	ND	0	0
50	no	ND	ND	0	0
51	yes	0	0	0	0
52	no	ND	ND	0	0
53	unknown	2	1	0	0
54	yes	6	6	6	6
55	yes	2	2	4	2
57	yes	4	3	4	4
58	yes	5	4	5	4
59	yes	3	2	2	2
60	yes	3	3	3	2
61	yes	5	4	6	5
62	no	ND	ND	0	0
63	no	ND	ND	0	0
64	yes	3	2	2	2
65	yes	3	2	2	2
66	yes	4	3	2	2
67	no	ND	ND	0	0
68	yes	3	3	2	1
69	yes	4	4	3	3
70	yes	3	3	3	3
71	yes	2	1	2	2
72	yes	7	7	7	7
73	yes	6	6	6	5
74	yes	0	0	1	1
75	no	2	2	3	3
76	yes	1	1	2	1
77	yes	2	1	2	1
78	yes	6	5	7	6
80	no	3	3	5	4
81	yes	6	6	7	6
82	no	ND	ND	0	0
83	yes	2	2	2	2
84	no	ND	ND	0	0
85	yes	2	2	3	2
86	yes	3	2	4	3
87	yes	3	2	3	2
88	no	ND	ND	0	0
89	no	ND	ND	0	0
90	yes	0	0	0	0
91	yes	6	5	5	5
92	no	ND	ND	0	0
93	yes	4	4	5	4
94	no	ND	ND	0	0
95	yes	2	1	2	2
96	yes	0	0	0	0
97	yes	5	5	5	4
98	no	ND	ND	0	0
99	no	0	0	0	0
100	yes	0	0	0	0
101	no	ND	ND	0	0
102	no	ND	ND	0	0
103	yes	6	5	5	5
104	yes	5	4	4	4
105	yes	5	4	4	3
106	no	ND	ND	0	0
107	yes	4	4	4	3
108	no	ND	ND	0	0
109	no	ND	ND	0	0
110	no	ND	ND	0	0
111	yes	6	5	4	4
112	no	ND	ND	0	0
113	yes	1	0	2	1
114	yes	1	1	1	1
115	no	6	6	4	4
116	no	ND	ND	0	0
117	unknown	6	5	4	3
118	yes	6	5	7	6
119	no	ND	ND	0	0
120	yes	1	1	1	1
121	yes	3	2	2	1
122	yes	4	3	3	3
123	no	NA	NA	1	1
124	yes	5	4	5	4
125	yes	4	3	3	2
126	yes	4	3	5	4
127	yes	3	2	2	2
128	no	ND	ND	0	0
129	yes	4	4	4	2
130	no	ND	ND	0	0
131	yes	2	2	2	2
132	yes	1	1	1	1
133	no	ND	ND	0	0
134	yes	5	3	4	3
135	no	ND	ND	0	0
136	yes	4	4	4	3
137	yes	1	1	2	1
138	no	ND	ND	0	0
139	no	ND	ND	0	0
140	yes	6	5	6	6
141	yes	4	4	5	5
142	no	ND	ND	0	0
143	yes	3	3	4	4
144	yes	5	4	5	4
145	yes	4	2	2	0
146	no	6	6	7	6
147	yes	0	0	0	0
148	yes	4	3	3	2
149	yes	5	5	5	4
150	unknown	0	0	0	0
151	no	ND	ND	0	0
152	yes	3	2	3	2
153	yes	1	0	0	0
154	yes	4	3	3	3
155	yes	1	1	6	5
156	yes	5	4	4	3
157	no	ND	ND	0	0
158	unknown	0	0	0	0
159	no	ND	ND	0	0
160	yes	1	1	3	2
161	no	ND	ND	0	0
162	yes	4	3	4	4
163	yes	4	4	3	3
164	yes	5	5	6	5
165	no	ND	ND	0	0
166	no	ND	ND	0	0
167	no	ND	ND	0	0
169	no	ND	ND	0	0
170	yes	5	3	5	4
171	yes	7	6	7	6
172	yes	0	0	0	0
173	yes	2	1	2	2
174	yes	2	1	2	0
175	yes	5	4	4	4
176	yes	5	4	5	4
177	yes	2	1	2	2
178	yes	1	1	1	0
179	yes	5	4	4	4
180	yes	2	2	2	1
181	yes	7	7	7	6
182	yes	2	1	2	0
183	yes	5	4	4	3
184	no	ND	ND	0	0
185	no	ND	ND	0	0
186	yes	4	4	5	4
188	yes	1	0	0	0
189	yes	6	5	5	4
190	no	ND	ND	0	0
191	no	ND	ND	0	0
192	no	ND	ND	2	0
194	no	ND	ND	0	0
195	yes	6	5	6	5
196	yes	3	2	4	3
197	yes	5	5	6	5
198	yes	2	1	6	5
199	yes	6	5	2	1
200	yes	2	2	2	2
201	yes	4	4	4	4
202	no	ND	ND	0	0
203	no	ND	ND	0	0
204	yes	4	3	3	3
205	no	ND	ND	0	0
206	no	ND	ND	0	0
207	yes	4	3	3	2
208	yes	6	5	5	4
209	no	1	1	5	4
210	yes	5	3	5	5
211	yes	3	2	2	1
212	yes	5	5	1	1
213	yes	4	3	4	3
214	no	NA	NA	1	1
215	yes	3	3	4	3
216	no	ND	ND	0	0
217	yes	4	3	3	2
218	yes	2	2	2	1
219	no	ND	ND	0	0
220	yes	4	2	3	2
221	yes	5	4	5	4
222	yes	4	4	4	4
223	no	ND	ND	0	0
224	no	ND	ND	0	0
225	yes	4	3	4	3
226	yes	3	3	4	4
227	yes	5	5	6	5
228	no	ND	ND	0	0
229	yes	4	4	4	3
230	unknown	0	0	1	1
231	yes	6	6	6	5
232	yes	0	0	0	0
233	yes	3	3	2	1
234	no	0	0	2	2
235	no	ND	ND	0	0
236	no	ND	ND	0	0
237	yes	5	5	6	5
238	no	ND	ND	0	0
239	yes	2	1	5	4
240	yes	1	1	1	1
241	no	ND	ND	0	0
242	yes	0	0	1	0
243	no	ND	ND	0	0
244	yes	0	0	0	0
245	yes	3	3	5	4
246	yes	3	3	4	4
247	yes	4	3	4	3
248	yes	0	0	0	0

Table 2. Raw data of self-reported history of TBE vaccination and TBEV-PRNT on day 0 and 28
A=Study participant ID,B=self-reported history of TBEV vaccination,C=NT80-TBEV day 0,D=NT90-TBEV day 0,E=NT80-TBEV day 28,F=NT90-TBEV day 28,ND=TBEV-PRNT not done,NA=serum not available.

		A TBEV vaccinated		B TBEV non-vaccinated		C Unknown TBEV vaccination status		D Total	
		n =	% of A % of D	n =	% of B % of D	n =	% C % of D	n =	% of D
		161	 66.5	**76**	 31.4	**5**	 2.1	**242**	100
NT-TBEV day 0	ND/NA	0		69	90.8 28.5	0		69	28.5
	0	17	10.6 7.0	1	1.3 0.4	3	60.0 1.2	21	8.7
	1	22	13.7 9.1	1	1.3 0.4	1	20.0 0.4	24	9.9
	2	23	14.3 9.5	2	2.6 0.8	0		25	10.3
	3	34	21.1 14.0	1	1.3 0.4	0		35	14.5
	4	33	20.5 13.6	1	1.3 0.4	0		34	14.0
	5	21	13.0 8.7	0		1	20.0 0.4	22	9.1
	6	8	5.0 3.3	1	1.3 0.4	0		9	3.7
	7	3	1.9 1.2	0		0		3	1.2
NT-TBEV day 28	ND/NA	0		0		0		0	
	0	21	13.0 8.7	67	88.2 27.7	3	60.0 1.2	91	37.6
	1	19	11.8 7.9	3	3.9 1.2	1	20.0 0.4	23	9.5
	2	28	17.4 11.6	1	1.3 0.4	0		29	12.0
	3	27	16.8 11.2	1	1.3 0.4	1	20.0 0.4	29	12.0
	4	34	21.1 14.0	3	3.9 1.2	0		37	15.3
	5	19	11.8 7.9	0		0		19	7.9
	6	10	6.2 4.1	1	1.3 0.4	0		11	4.5
	7	3	1.9 1.2	0		0		3	1.2

Table 3. Absolute data and proportions of counts NT-TBEV.
Shown are the absolute and relative proportions of NT90-TBEV of study participants with, without and unknown self-reported history of TBE vaccination and the overall cohort**.**

5.2. Data of YFV-FluoRNT results by Lisa Lehmann

A	B	C	D	E
1	-3.1379866	-2.7660695	-3.6782781	-3.3719445
2	-2.94445	-2.6160983	-2.9007896	-2.5946364
3	-3.0277898	-2.6283221	-3.3658273	-3.0407798
4	-3.1521891	-2.7870165	-3.251502	-2.9183384
5	NA	NA	NA	NA
6	NA	NA	NA	NA
7	-3.1423731	-2.7863815	-3.3321141	-3.0157467
8	-2.5305805	-2.2217503	-3.5415034	-3.2623304
9	-1.9837402	-1.6742959	-2.9458575	-2.6119257
10	-2.5073286	-2.1181942	-2.6681504	-2.338133
11	-2.8024742	-2.4484174	-2.9828769	-2.6281423
12	-2.4970371	-2.0839954	-2.6401856	-2.2362292
13	-3.1370389	-2.8463591	-3.1742025	-2.8895324
14	-3.0093384	-2.5947877	-3.9276684	-3.6288833
15	-2.3991564	-2.0666364	-3.1193585	-2.8488869
16	-3.1569123	-2.73845	-3.4773461	-3.1689603
17	-2.7487879	-2.2643022	-3.0014411	-2.6920164
18	-2.7132995	-2.2683149	-2.8182443	-2.480122
19	-3.1049556	-2.7273649	-3.4765449	-3.1327607
20	-3.1142704	-2.7472282	-3.1599858	-2.8192522
21	-2.9654249	-2.6425841	-3.3054997	-3.0331367
22	-2.2791072	-1.8710982	-3.2177485	-2.9196945
23	-3.1337007	-2.618422	-2.9008892	-2.4751287
24	-2.5693097	-2.1202749	-3.3860331	-2.9714935
25	-3.457504	-3.0592449	-3.4244932	-3.0731736
26	-2.4573126	-2.0134134	-3.2413901	-2.924469
27	-2.7634304	-2.3890445	-3.3986237	-3.0509905
28	-3.111924	-2.7638727	-3.2378061	-2.9536046
29	-2.7580071	-2.3871842	-3.190808	-2.8427155
30	-2.4172916	-1.9156338	-3.3061245	-2.9566614
31	-3.1977678	-2.8042153	-3.1353347	-2.7332283
32	NA	NA	-3.4632919	-3.1717979
33	-3.3113037	-2.900461	-3.3756308	-3.0364724
34	-3.1849505	-2.8928626	-3.157833	-2.8822166
35	-2.3219296	-1.8820495	-2.8506412	-2.4771395
36	-2.8327907	-2.4429262	-2.8538728	-2.5289803
37	-2.6550538	-2.3124016	-2.48816	-2.1740447
38	-2.7501771	-2.4248957	-3.0113557	-2.7101407
39	-3.415774	-3.0227697	-3.5301969	-3.2173135
40	NA	NA	-3.2938417	-2.9133607
41	-2.7692907	-2.3790244	-2.9297093	-2.5385985
42	-2.7501771	-2.4248957	-3.0113557	-2.7101407
43	-2.8418781	-2.5785644	-2.8980486	-2.6437347
44	-2.6613521	-2.3188026	-3.1003075	-2.7501678
45	-2.6651379	-2.3211382	-2.5314285	-2.1968158
46	-3.3361116	-2.8767131	-3.2859641	-2.8796371
47	-3.3395289	-2.8224811	-3.4731173	-3.0771105
48	-3.1932024	-2.8445306	-3.0894082	-2.8410844
49	-2.8560745	-2.5461495	-2.9262181	-2.6342479
50	-2.9598711	-2.586648	-2.8807112	-2.562594
51	-2.3922413	-2.0633218	-3.3581431	-2.0566648
52	-3.4318932	-3.0643314	-3.5188735	-3.1953866
53	-2.8056549	-2.4406372	-2.8977607	-2.4900819
54	-3.1644105	-2.9012123	-3.3095274	-3.0073239
55	-3.2782586	-3.0081057	-3.2479079	-2.9797408
56	-2.9273377	-2.591441	-3.359729	-3.0905322
57	-2.4715175	-2.1516771	-2.465656	-2.1564205
58	-2.863765	-2.5889603	-2.8216296	-2.5250614
59	-2.7599462	-2.4660412	-2.7863677	-2.5055568
60	-3.0900042	-2.748919	-2.9561426	-2.6388003
61	-2.5137669	-2.2655372	-2.5369585	-2.2845345
62	-2.6604781	-2.2252283	-2.9413225	-2.5644235
63	-2.2590714	-1.9761398	-2.2906576	-2.0033624
64	-3.463136	-3.2248617	-3.4986807	-3.2848577
65	-2.5054544	-2.0614877	-2.860741	-2.5131317
66	-2.6380314	-2.3581081	-2.855736	-2.5532557
67	-2.7336704	-2.3344994	-3.4068419	-3.0507239
68	-3.1514584	-2.8233066	-3.3330254	-3.2127427
69	-2.3537228	-2.0143103	-2.7981551	-2.4823806
70	-2.82949	-2.4885739	-2.7719622	-2.4723415
71	-2.7193491	-2.3987129	-3.0841442	-2.7946857
72	-2.7638727	-2.3378061	-2.9536046	-2.5536046
73	-2.9357908	-2.548255	-3.248041	-2.8840175
74	-2.4190037	-2.0510995	-2.9869212	-2.633966
75	-1.9938686	-1.6603242	-3.6222232	-3.274772
76	-2.8738891	-2.5690004	-2.859778	-2.5869979
77	-2.620484	-2.3409634	-2.7674991	-2.4843292
78	-3.8491904	-3.5102717	-3.7224577	-3.3954123
79	-2.5961466	-2.2751284	-3.046945	-2.743361
80	-2.7966306	-2.469387	-3.2910862	-2.9528514
81	-3.1023502	-2.8133472	-2.9041303	-2.6305205
82	-3.1530185	-2.8681493	-3.0694711	-2.7815624
83	-2.5455367	-2.2439631	-3.117163	-2.8360283
84	-3.1403506	-2.7975026	-3.4915366	-3.2260664
85	-2.5126435	-2.2403813	-2.7978777	-2.5035175
86	-2.2282626	-1.815534	-2.7952742	-2.4357749
87	-2.6613521	-2.3188026	-3.1003075	-2.7501678
88	-2.5457539	-2.2046519	-3.1228086	-2.6950055
89	-3.291499	-2.8484435	-3.3710854	-3.0112863
90	-2.6929989	-2.3884426	-2.719564	-2.3881198
91	-3.2753921	-2.9023401	-3.5914858	-3.2537303
92	-2.65683	-2.33089	-2.4712906	-2.176441
93	-2.3384459	-1.951497	-2.6640979	-2.3263149
94	-2.5946959	-2.1929815	-2.7571443	-2.3852118
95	-2.4498227	-2.0827002	-3.1892524	-2.7967821
96	-2.9596144	-2.5062366	-3.2102508	-2.8355775
97	-2.7601479	-2.42845	-2.8308294	-2.5350998
98	-2.8231851	-2.3780208	-3.2832262	-2.8400369
99	-2.9413855	-2.630421	-3.4239365	-3.0887872
100	-2.3537107	-1.8261169	-3.4643589	-3.1513999
101	-3.047926	-2.7882584	-3.5551472	-3.2544043
102	-2.647128	-2.3319367	-3.4625756	-3.1358216
103	-2.714194	-2.4022132	-3.2675247	-2.9665327
104	-2.4790397	-2.1512827	-2.8837565	-2.5824366
105	-2.1096334	-1.6450823	-2.9116784	-2.5708654
106	-3.2473507	-2.9158215	-2.9972755	-2.7262226
107	-3.0730045	-2.7112558	-3.0038705	-2.6951882
108	-2.7868016	-2.3150114	-2.9286139	-2.4659286
109	-3.0655071	-2.7007221	-3.0239357	-2.7132512
110	-2.9773887	-2.4785979	-2.9280941	-2.5308662
111	-2.481272	-2.1093657	-3.2480694	-2.9606673
112	-2.9928104	-2.5682059	-2.9522589	-2.5720953
113	-3.1318254	-2.7288311	-3.0108015	-2.667302
114	-2.6767404	-2.3449989	-3.0323267	-2.7628884
115	-3.1383036	-2.804005	-4.1059811	-3.8053354
116	-3.2481127	-2.7684611	-3.3964152	-3.0150116
117	-3.5539147	-3.1206515	-4.1992299	-3.8506041
118	-2.7348506	-2.4161695	-2.9590593	-2.6425835
119	-2.9357716	-2.5900557	-3.3113892	-2.9964756
120	-2.5853773	-2.2514257	-3.2162991	-2.9381927
121	-2.5087792	-2.2028982	-2.831942	-2.5811033
122	-2.6826995	-2.3075739	-3.5852744	-3.2472262
123	-3.0519979	-2.6394886	-3.3861758	-3.0089468
124	-2.4140031	-2.1296537	-2.1314172	-1.8693595
125	-2.5470573	-2.256292	-3.4078021	-3.0773713
126	-2.7320604	-2.4557647	-2.6807899	-2.4129359
127	-2.8126651	-2.4894041	-3.518209	-3.2025576
128	-2.6210927	-2.277292	-2.8290016	-2.5450473
129	-2.5845963	-2.2380414	-2.8924413	-2.5464168
130	-2.9301169	-2.6207228	-3.3297583	-2.9552739
131	-2.6905613	-2.3477497	-3.0559371	-2.7110171
132	-2.4218095	-2.1474866	-3.1400632	-2.8688229
133	-2.5412438	ND	-3.1865059	-1.7051603
134	-2.7955008	-2.4716337	-3.0461365	-2.799109
135	-2.6007734	-2.3480494	-2.4879516	-2.2052209
136	-2.7449236	-2.2876172	-3.3891401	-2.9651342
137	-3.1062315	-2.7842094	-3.3462108	-3.0282364
138	-2.646685	-2.2926614	-2.5894889	-2.2620517
139	-2.6573608	-2.3004715	-3.2707726	-2.8939721
140	-2.8394987	-2.5238957	-3.180791	-2.8577331
141	-3.140886	-2.7823913	-3.4574116	-3.1504178
142	-2.4719368	-2.1248069	-3.0865834	-2.8008114
143	-2.9260493	-2.5781827	-2.9062907	-2.5786554
144	-2.6857265	-2.2135313	-3.609671	-3.2473698
145	-3.2253873	-2.9318604	-3.4204489	-3.1441708
146	-2.9446572	-2.5933717	-3.2436098	-2.9027544
147	-2.7911208	-2.451268	-2.8933141	-2.6344367
148	-3.4443451	-3.1284305	-3.3582936	-3.0865493
149	-2.948087	-2.5953114	-3.0979797	-2.7968067
150	-2.5873019	-2.3333353	-3.3615034	-3.1912347
151	-2.8147999	-2.5068359	-3.4726212	-3.1482842
152	-2.5962286	-2.2991564	-2.5648048	-2.2865326
153	-2.8559713	-2.4632623	-3.6109765	-3.3565809
154	-3.1032103	-2.890259	-3.3373138	-3.0346725
155	-2.8951134	-2.6004967	-3.1953615	-2.9390033
156	-3.202303	-2.8723873	-3.5035365	-3.2098192
157	-2.9096507	-2.6048034	-3.1058963	-2.8196826
158	-3.4805493	-3.1600005	-3.3616368	-3.0604711
159	-3.0389737	-2.7505308	-3.2974297	-3.0124405
160	-3.1334401	-2.8316526	-3.2762183	-3.0116249
161	-3.4815654	-3.1633876	-3.4300351	-3.1735994
162	-3.1589071	-2.8530044	-3.7076884	-3.3995061
163	-3.0852135	-2.7644911	-3.3815283	-3.0467905
164	-2.1516702	-1.9143704	-3.8685832	-3.6141032
165	-3.2426179	-2.7925268	-3.7195236	-3.4256125
166	-4.0455947	-3.3835924	-3.5263424	-3.3271885
167	-3.1471277	-2.8168888	-3.4660797	-3.1881884
168	-2.8983772	-2.5929074	-3.4845502	-3.2664567
169	-2.8983772	-2.5929074	-3.4845502	-3.2664567
170	-2.9166622	-2.6693476	-2.9386314	-2.6153613
171	-3.0253493	-2.6537689	-3.5785045	-3.2237106
172	-3.3468336	-3.0281623	-3.226747	-2.9377776
173	-2.7057174	-2.4142742	-2.965081	-2.6987976
174	-2.6520474	-2.3431564	-2.5959858	-2.3151535
175	-2.8452186	-2.5692327	-2.8472524	-2.5951173
176	-2.967352	-2.7008485	-3.5285516	-3.2242022
177	-2.6413574	-2.3157424	-3.1355485	-2.8288254
178	-3.0905923	-2.7743355	-3.0905923	-2.7743355
179	-2.8353349	-2.5179963	-3.4211311	-3.0797696
180	-2.9021973	-2.586382	-2.7638553	-2.4835174
181	-3.0231688	-2.735645	-3.2860433	-3.0189534
182	-3.381865	-3.0917924	-3.4856864	-3.1737576
183	-2.8362675	-2.4871663	-3.2749303	-2.9736063
184	-3.1841822	-2.8490737	-3.2917256	-2.9801515
185	-3.0887137	-2.8283183	-3.1183641	-2.8753714
186	-3.1175564	-2.7984245	-3.7161727	-3.4744889
187	-3.7027342	-3.3480938	-3.7018732	-3.3656457
188	-2.8955998	-2.5633447	-3.2221845	-2.9100056
189	-2.8320642	-2.5067233	-3.2265816	-2.8067785
190	-2.9072106	-2.5944638	-2.9588368	-2.6709016
191	-3.2717945	-2.9629182	-3.1970879	-2.9248949
192	-2.8283602	-2.4685629	-3.1466184	-2.8785021
193	-2.8695595	-2.4479614	-3.2776793	-2.8039837
194	-2.8904973	-2.5446361	-3.4910059	-3.151899
195	-3.7270952	-3.4110982	-3.6951117	-3.4202285
196	-2.9063096	-2.6271123	-3.6752408	-3.4289009
197	-2.8501908	-2.5775675	-2.8071585	-2.5405072
198	-2.6703504	-2.3375601	-3.6541545	-3.3868447
199	-3.0183847	-2.7164417	-3.1412233	-2.834524
200	-2.8046778	-2.4980312	-2.8914352	-2.5465142
201	NA	NA	NA	NA
202	-4.3009992	-3.9083238	-4.0533922	-3.6791729
203	-2.4408391	-2.1039548	-3.1812726	-2.8633618
204	-2.9195699	-2.5860379	-3.2263552	-2.9245137
205	-2.7893235	-2.1907639	-3.5958199	-3.2907122
206	-2.6774279	-1.9748295	-2.7688605	-2.0091358
207	-2.1239823	-1.8189458	-2.0919591	-1.7724399
208	-2.4238874	-2.1410695	-3.044985	-2.7926735
209	-2.7893235	-2.1907639	-3.5958199	-3.2907122
210	-3.3523757	-3.1118554	-3.6174511	-3.3648389
211	-2.5446248	-2.2943586	-2.9433186	-2.6758432
212	-3.3915905	-3.0952644	-3.5096508	-3.1789813
213	-3.1128074	-2.8385815	-2.8794212	-2.5645848
214	-3.9161413	-2.6084776	-3.2398509	-2.9562245
215	-3.4000797	-3.0908932	-3.9168886	-3.6571904
216	-2.8983772	-2.5929074	-3.4845502	-3.2664567
217	-3.1465066	-2.8588072	-3.0163388	-2.7349077
218	-3.390381	-3.1124826	-3.3449153	-3.0632732
219	-3.450847	-3.1472794	-3.2959084	-3.0090486
220	-3.2361507	-2.9069111	-3.6851108	-3.4249799
221	-3.4720947	-3.1524689	-3.7048128	-3.4078786
222	-3.1596594	-2.8836761	-3.0903929	-2.8306635
223	-2.894475	-2.6319533	-3.6559829	-3.4133563
224	-3.2415835	-2.9361197	-3.3919182	-3.0840494
225	-1.6825893	-1.1149848	-3.16957	-2.8827808
226	-3.1801061	-2.8981222	-3.7007338	-3.3718518
227	-3.3877197	-3.0722671	-3.4635726	-3.2045561
228	-3.2881254	-3.031153	-3.8648318	-3.5839224
229	-2.7016406	-2.3515922	-2.7995025	-2.4737862
230	-3.2106496	-2.9800209	-3.6827545	-3.4179934
231	-3.2788368	-3.0430947	-3.1329223	-2.8963828
232	NA	NA	-2.7726197	NA
233	-3.319124	-2.8462469	-3.449299	-3.112691
234	NA	NA	-3.1218592	-2.7185239
235	-2.6992256	-2.3829134	-3.2098311	-2.9339079
236	-2.5130266	-2.1627184	-3.1583438	-2.8431363
237	-2.7963469	-2.4695583	-3.1823333	-2.8303282
238	-1.9678456	-1.5795744	-2.3730038	-2.0188103
239	-2.3259447	-1.9540552	-2.9777374	-2.6764935
240	-3.2445385	-2.9244168	-3.1239448	-2.8877246
241	-3.2191143	-2.8533437	-3.2782208	-2.9533006
242	-3.6792001	-3.3704988	-3.2714331	-2.9837336
243	-3.0499706	-2.7825396	-3.141185	-2.9284169
244	-3.390103	-2.9787174	-3.6429998	-3.3725871
245	-3.0844172	-2.8192397	-3.3437431	-3.0428841
246	-2.6884689	-2.415372	-3.0271754	-2.7782369
247	-3.0423005	-2.7650126	-3.3240019	-3.0075879
248	-2.7867339	-2.4980081	-2.7970726	-2.5286765

Table 4. Raw data of YFV-FluoRNT on day 14 and 28.
A= study participant ID, B= NT80-YFV day 14, C= NT90-YFV day 14, D= NT80-YFV titer day 28, E= NT90-YFV titer day 28. ND= YFV-FluoRNT not done, NA= serum not available.

5.3. Data of IIFT results

IIFT ID	TBEV-PRNT-90 ID	NT-TBEV day 0	NT-TBEV day 28	TBEV vaccination	IIFT IgM day 0							IIFT IgM day 28							IIFT IgG day 0							IIFT IgG day 28						
					TBEV	WNV	JEV	YFV	DV1	DV2	DV3	DV4	ZKV	TBEV	WNV	JEV	YFV	DV1	DV2	DV3	DV4	ZKV	TBEV	WNV	JEV	YFV	DV1	DV2	DV3	DV4	ZKV
1	192	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
2	228	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	235	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	236	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	238	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	241	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	31	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	41	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	109	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	110	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	112	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
12	119	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	138	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	151	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	166	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	184	ND	0	no	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	211	2	1	yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1	1	0	0	0	0	0	0	0
18	233	3	1	yes	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0
19	240	1	1	yes	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	6	0	0	2	1	0	2	0	0	0
20	217	3	2	yes	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0
21	220	2	2	yes	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	9	5	6	5	6	7	6	6	6	7
22	199	5	1	yes	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	10	5	5	5	5	6	5	5	6	6
23	213	3	3	yes	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	10	4	6	10	7	7	5	6	5	5
24	225	3	3	yes	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	9	5	6	5	6	7	6	5	6	6
25	229	4	3	yes	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0
26	25	1	4	yes	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	7	3	4	3	4	5	4	4	4	3
27	221	4	4	yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	6	6	3	6	7	6	6	7	4
28	226	3	4	yes	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	8	3	4	1	4	5	3	3	3	3
29	239	1	4	yes	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	6	0	0	0	0	0	0	0	0	1
30	198	1	5	yes	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	5	0	0	0	0	0	1	0	0	0
31	227	5	5	yes	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	9	5	7	4	4	5	4	4	4	3
32	231	6	5	yes	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	10	6	6	6	7	7	6	7	5	5
33	237	5	5	yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	8	8	8	10	10	8	8	6	6
34	8	6	6	yes	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	10	8	9	9	7	8	7	8	6	6
35	14	6	6	yes	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	9	8	8	8	8	9	8	8	8	8
36	54	6	6	yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	6	7	7	7	7	7	7	7	5
37	81	6	6	yes	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0
38	171	6	6	yes	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	9	10	10	10	10	10	10	0	2	2
39	28	7	7	yes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	8	8	5	6	8	7	7	5	5
40	72	7	7	yes	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	9	0	2	0	10	10	10	10	0	0

Table 5. Raw data of IIFT results.
ND = test not done; 0 = no antibody titre detectable; antibody titre at dilution 1=1:10, 2=1:20, 3=1:40, 4=1:80, 5=1:160, 6=1:320, 7=1:640, 8= 1:1280, 9=1:2560, 10=1:5120

5.4. Documented vaccination schedules

Study ID	month.year: type of TBEV vaccination		Study ID	month.year: type of TBEV vaccination	
	Basic immunisation	Booster immunisations		Basic immunisation	Booster immunisations
211	04.2007: Encepur® Kinder 04.2007: Encepur® Kinder 05.2007: Encepur® Kinder	07.2012: Encepur®	229	03.2017: FSME-IMMUN®	
213	04.2007: FSME-IMMUN® Junior 05.2007: FSME-IMMUN® Junior 04.2008: FSME-IMMUN® Junior	06.2014: FSME-IMMUN®	231	05.2007: Encepur® Kinder 06.2007: Encepur® Kinder 05.2008: FSME-IMMUN® Junior	07.2013: Encepur® 09.2018: Encepur®
217	06.2007: Encepur® 07.2007: Encepur® 06.2008: Encepur®	06.2014: FSME-IMMUN®	232	08.2006: Encepur®	
220	05.1999: FSME-IMMUN® 05.1999: FSME-IMMUN® 03.2000: Tico Vac	03.2003: FSME-IMMUN® 05.2008: FSME-IMMUN® 03.2015: FSME-IMMUN®	233	09.1992: Encepur® Kinder 08.1993: FSME-IMMUN®	07.1999: FSME-IMMUN® 03.2004: FSME-IMMUN® 06.2008: Encepur® 05.2012: Encepur®
221	03.1996: FSME-IMMUN® 04.1996: FSME-IMMUN® 04.1997: FSME-IMMUN®	05.2001: FSME-IMMUN® 05.2006: FSME-IMMUN® 04.2011: Encepur®	237	08.2005: FSME-IMMUN® Kinder 09.2005: FSME-IMMUN® Kinder 07.2007: Encepur® Kinder	05.2009: FSME-IMMUN® Junior 07.2014: FSME-IMMUN®
225	04.1999: FSME-IMMUN® 05.1999: FSME-IMMUN® 11.1999: Encepur®	03.2009: FSME-IMMUN® 06.2013: FSME-IMMUN® 12.2017: FSME-IMMUN®	239	12.2007: FSME-IMMUN® Junior 01.2008: FSME-IMMUN® Junior 03.2009: FSME-IMMUN® Junior	02.2015: FSME-IMMUN®
226	06.2003: Encepur® Kinder 09.2003: FSME-IMMUN® Junior	09.2016: FSME-IMMUN® 10.2016: FSME-IMMUN® 04.2017: FSME-IMMUN®	240	08.2017: FSME-IMMUN® 09.2017: FSME-IMMUN® 05.2018: FSME-IMMUN®	
227	06.2002: Encepur® Kinder 07.2002: Encepur® Kinder 05.2003: Encepur® Kinder	07.2007: Encepur® Kinder 10.2012: FSME-IMMUN® 10.2017: Encepur®	244	09.2005: Encepur® Kinder 11.2005: Encepur® Kinder 10.2006: Encepur® Kinder	

Table 6. Exact vaccination schedules of TBE immunized selected for IIFT.
For only n=16 study participants` exact TBEV vaccination schedules were documented.

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Prior flavivirus immunity skews the yellow fever vaccine response to cross-reactive antibodies with potential to enhance dengue virus infection (1).

Antonio Santos-Peral[1], Fabian Luppa[2], Sebastian Goresch[1], Elena Nikolova[1], Magdalena Zaucha[1], Lisa Lehmann[1], Frank Dahlstroem[1], Hadi Karimzadeh[1], Julia Thorn-Seshold[1,3], Elena Winheim[4], Ev-Marie Schuster[5], Gerhard Dobler[6], Michael Hoelscher[2,7,8], Beate M Kümmerer[9,10], Stefan Endres[1,11], Kilian Schober[5], Anne B Krug[4], Michael Pritsch[2], Giovanna Barba-Spaeth[12*], Simon Rothenfusser[1,11*]. Prior flavivirus immunity skews the yellow fever vaccine response to cross-reactive antibodies with potential to enhance dengue virus infection. Nat Commun 15, 1696 (2024). https://doi.org/10.1038/s41467-024-45806-x

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