# Salivary SARS-CoV-2 antibodies for assessing infection exposure and vaccine response

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## Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

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Finally, I would like to say thank you all from my heart!

# Contents

# List of abbreviations

| | |
|---|---|
| ACE2 | Angiotensin-converting enzyme 2 |
| ARDS | Acute respiratory distress syndrome |
| CDC | Centers for Disease Control and Prevention |
| COVID-19 | Coronavirus disease 2019 |
| ELISA | Enzyme-linked Immunosorbent Assay |
| EIA | Enzyme immune assay |
| MERS-CoV | Middle East Respiratory Syndrome Coronavirus |
| MIS-C | Multisystem inflammatory syndrome in children |
| M protein | Membrane protein |
| NCP, N protein | Nucleocapsid protein |
| NTD | N-terminal domain |
| NSP | Non-structural protein |
| PRNT | Plaque reduction neutralization test |
| PSO | Post-symptom onset |
| RBD | Receptor-binding domain |
| RT-qPCR | Real-time reverse transcription-polymerase chain reaction |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus type 2 |
| SIgA | Secretory IgA |
| S Protein | Spike protein |
| TMPRSS2 | Transmembrane protease serine subtype 2 |
| Tri | Trimer (of the spike protein) |
| VOI | Variants of interest |
| VOC | Variants of concern |
| WT | Wild type (ancestral strain) |

# Zusammenfassung

Zu Beginn der Pandemie gab es nur begrenzte Informationen zur Krankheitslast, Verbreitung und Immunantwort von SARS-CoV-2 Infektionen bei Kindern und Jugendlichen. Der Schwerpunkt dieser Arbeit liegt in der Untersuchung von SARS-CoV-2 spezifischen IgG-Antikörpern zur retrospektiven Bestimmung der Prävalenz von SARS-CoV-2 bei Kindern und Jugendlichen. Hierfür wurde schrittweise ein ELISA Test entwickelt, welcher hochsensitiv und spezifisch RBD IgG im Speichel detektiert. IgG-Antikörper diffundieren vom Plasma in den Speichel und die IgG-Konzentrationen korrelieren zuverlässig. Antikörper im Speichel sind ein essenzieller erster Schutz des Immunsystems gegen das SARS-CoV-2 Virus. Zudem ist die Speichelsammlung nicht-invasiv und eignet sich sehr gut für bevölkerungsbasierte Studien, insbesondere für die Beprobung von Kindern und Jugendlichen.

Nach der Optimierung des ELISAs und Etablierung mit Kontrollproben wurde eine gute Sensitivität (87 %) bei maximierter Spezifität erreicht. Der Assay wurde in der longitudinalen und prospektiven Beobachtungsstudie zur Seroprävalenz von SARS-CoV-2 Infektionen bei Kindern und Jugendlichen in Tübingen („Coro-Buddy“) angewandt. Insgesamt wurden 2069 Kinder und Jugendliche eingeschlossen und wiederholt untersucht. Im ersten Jahr der Pandemie (2020) wurden im Stadtgebiet Tübingen in der Untergruppe der 1- bis 10-Jährigen, 837 Kinder eingeschlossen (12 % der Population dieser Altersgruppe) und wiederholt Speichelproben genommen. Insgesamt war die Exposition gegenüber SARS-CoV-2 in dieser Alterskohorte im ersten Pandemiejahr mit 1,6 % relativ gering, aber dreimal höher als die 0,5 % PCR-positiven Kinder gleichen Alters, die vom Gesundheitsamt erfasst wurden - was unter anderem auf die symptombezogene Teststrategie zurückzuführen ist. Die Prävalenz von SARS-CoV-2-Antikörpern war bei den Kindergartenkindern mit 0,4 % deutlich niedriger als bei den Grundschulkindern mit 3,0 %. Dies gibt Hinweise darauf, dass jüngere Kinder seltener mit der Wildtyp-Variante von SARS-CoV-2 infiziert waren, obwohl beide Altersklassen Lockdown-bedingt größtenteils nicht in ihren Bildungseinrichtungen waren.

Weitere Untersuchungen an Erwachsenen haben gezeigt, dass eine milde Infektion mit SARS-CoV-2 (Wildtyp) zu einer über ein Jahr anhaltenden Antikörperantwort in Plasma und Speichel führen kann. Mit der Zeit nahmen die Antikörper jedoch ab,

sodass der Schutz vor einer SARS-CoV-2-Infektion wahrscheinlich reduziert war. Eine nachfolgende Impfung mit COVID-19 führte zu einem starken Anstieg der Antikörperspiegel sowohl im Speichel als auch im Blut (46-fach bzw. 72-fach, einen Monat nach der Impfung). Darüber hinaus war die Neutralisationswirkung im Plasma bei den geimpften Personen deutlich höher als bei der Rekonvaleszenz-Kohorte.

Mit dem Auftreten der Omikron-Variante Ende 2021 veränderte sich das Pandemiegeschehen erheblich und die Infektionsraten stiegen weltweit deutlich an. Unerwarteterweise wurde ein ausgeprägter Unterschied in der IgG Immunantwort zwischen den mit Prä-Omikron-Varianten und den mit der Omikron-Variante Infizierten festgestellt. Viele Kinder, die zum ersten Mal mit SARS-CoV-2 infiziert waren, hatten nach einer Omikron-Infektion keine RBD IgG-Antikörper im Speichel und im Plasma (141/173). Vergleichbares wurde auch für weitere Antigene (Spike Trimer und NCP) festgestellt. Diese Ergebnisse deuten auf eine Veränderung der Immunantwort durch Omikron hin, die sich in einer verminderten Antikörperantwort äußert. Eine Folge könnte ein erhöhtes Infektionsrisiko durch zukünftige SARS-CoV-2-Varianten sein.

Zusammenfassend lässt sich sagen, dass der IgG-ELISA auf Speichelbasis für die Bestimmung der Immunantwort und der SARS-CoV-2-Prävalenz im großen Maßstab sehr nützlich war. Weitere Untersuchungen der zellulären Immunantwort sind erforderlich, um das Verständnis der Immunität zu vervollständigen. Insbesondere im Hinblick auf neue SARS-CoV-2-Varianten und andere Pandemien leisten Speichel-basierte ELISAs einen wichtigen Beitrag zum Verständnis des Pandemiegeschehens.

# Abstract

At the beginning of the SARS-COV-2 pandemic, limited information regarding disease burden, prevalence and immune response was available in the pediatric population. This thesis investigated SARS-CoV-2 specific IgG antibodies for the retrospective analysis of SARS-CoV-2 exposure in children. For this purpose, a sensitive and specific ELISA was developed to detect RBD-specific IgG antibodies in saliva. IgG antibodies diffuse from plasma into saliva, resulting in correlating IgG levels. Further, salivary antibodies are crucial for the immune response as they form the first line of defense against the SARS-CoV-2 virus. The saliva collection is simple and non-invasive, which makes it suitable for population-based studies, particularly for sample collections with children and adolescents.

After the optimization of the ELISA and establishment of the control samples, the assay displayed a high sensitivity (87%) with maximized specificity. This assay was implemented in a prospective, longitudinal, observational study ("Coro-Buddy") measuring the seroprevalence of children and youths in Tübingen. Overall, 2069 children and adolescents were included and repeatedly sampled. In the first year of the pandemic in 2020, 837 children aged 1 to 10 years old (12% of the population of that age group in the city district of Tübingen) were included in a substudy, with saliva samples being repeatedly collected. The overall SARS-CoV-2 exposure in 2020 for this age cohort was comparably low at 1.6% but 3 times higher than the registered PCR positivity of 0.5% for the respective age group. This discrepancy might be linked to a widely used symptom-based test strategy. A substantial difference in prevalence was observed between preschool and primary school children of 0.4% and 3.0%, respectively. There is evidence that young children were less often infected with the SARS-CoV-2 wild-type variant than older population groups, even though the respective educational facilities were closed for an extended time due to the lockdown.

Additional experiments showed that mild SARS-CoV-2 wild-type infections in adults can induce a long-lasting antibody response that can persist over one year in plasma and saliva. However, antibodies were waning over time, potentially lowering the protection against SARS-CoV-2 infection. A subsequent COVID-19 vaccination boosted IgG levels tremendously in both specimens (plasma 72-fold and saliva 46-fold, one month post-vaccination). Furthermore, vaccinees showed greatly enhanced neutralization activity compared to the non-vaccinated, convalescent cohort.

With the emergence of the Omicron variant in 2021, the progression of the pandemic changed, and the number of infected cases rose rapidly worldwide. Unexpectedly, a profound difference in the antibody expression between pre-Omicron and Omicron convalescent youths was found. Most children exposed to SARS-CoV-2 for the first time developed, after an Omicron infection, no RBD-specific IgG antibodies in saliva (141 of 173) and plasma. A similar phenomenon was observed for other antigens (trimeric Spike and NCP). Altogether, there is strong evidence that the immune system was altered by the Omicron variant, leading to a reduced antibody response in youths. Potentially, this could lead to an increased risk of reinfection by newly emerging SARS-CoV-2 variants.

In summary, the saliva-based ELISA has proven to be a powerful tool to investigate the immune response and SARS-CoV-2 prevalence in a large study cohort. Further research on the cellular immune response in children will be necessary for a more comprehensive understanding of immunity. Regarding future applications, saliva-based assays can contribute much by examining the prevalence and immune response in population-based studies, especially considering new upcoming SARS-CoV-2 variants and other pandemics.

# List of publications

Published articles in peer-reviewed scientific journals:

**Non-Invasive Antibody Assessment in Saliva to Determine SARS-CoV-2 Exposure in Young Children**

Heinzel C*, Pinilla Y*, Elsner K, Friessinger E, Mordmüller B, Kremsner PG, Held J, Fendel R, & Kreidenweiss

Frontiers in Immunology (2021) 12; (*shared authorship)

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**SARS-CoV-2 Antibodies Are Persisting in Saliva for More Than 15 Months After Infection and Become Strongly Boosted After Vaccination**

Pinilla Y*, Heinzel C*, Caminada L, Consolaro D, Esen M, Kremsner P G, Held J, Kreidenweiss A, & Fendel R

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**Saliva and Plasma Antibody Levels in Children and Adolescents After Primary Infection With Omicron Variants of SARS-CoV-2 Infection in Germany**

Heinzel C, Pinilla Y, Binder A, Kremsner P G, Held J, Fendel R, Kreidenweiss A

JAMA Pediatrics (2023) 177(6) 640-641

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The following articles (with co-authorship) were published in peer-reviewed scientific journals during the thesis but not included:

**First-in-human use of a modular capsid virus-like vaccine platform: an open-label, non-randomised, phase 1 clinical trial of the SARS-CoV-2 vaccine ABNCoV2**

Smit M, Sander A, […] Vidal-Calvo E; The Lancet Microbe (2023) 4(3) e140-e148

**Antibody Binding and Angiotensin-Converting Enzyme 2 Binding Inhibition Is Significantly Reduced for Both the BA.1 and BA.2 Omicron Variants**

Junker D, Becker M, […] Schneiderhan-Marra N; Clinical Infectious Diseases (2023) 76(3) e240-e249

**Immune response to SARS-CoV-2 variants of concern in vaccinated individuals**

Becker M, Dulovic A, […] Schneiderhan-Marra N, Nature Communications 2021 12:1 (2021) 12(1) 1-8

**Complementary methods for SARS-CoV-2 diagnosis in times of material shortage**

Sandri T, Inoue J, […] Kreidenweiss A; Scientific Reports 2021 11:1 (2021) 11(1) 1-8

Declaration of own contributions to the collaborative publications:

The percentiles of my contributions were summarized in the provided document for collaborative publications.

In the publication “Non-Invasive Antibody Assessment in Saliva to Determine SARS-CoV-2 Exposure in Young Children”, I have contributed to the following parts:

- Development and implementation of a plasma and saliva-based, RBD-specific IgG ELISA. This includes the SARS-CoV-2 antigen generation and measurement of the Coro-Buddy study samples. This work was performed with

Karolin Lenckner, Käthe Elsner, Nina Huber, Martha Klug and Johanna Griesbaum. Experiment planning was shared with Rolf Fendel.

- Study design, organization, saliva specimen collection and sample preparation. This work was shared and mostly performed by Yudi Tatiana Pinilla (Study organization and design), Evelyn Friessinger, Johanna Griesbaum (sample preparation), Andrea Kreidenweiss, Rolf Fendel and Jana Held.
- Data analysis, interpretation, graphic generation and statistics. This work was shared with Yudi T. Pinilla, Rolf Fendel and Douglas Consolaro.
- The writing of the manuscript draft (figure descriptions, parts of the methodology and results section) and proofreading. These tasks were mainly performed by Andrea Kreidenweiss, Yudi T. Pinilla and Rolf Fendel.

In the publication “SARS-CoV-2 Antibodies Are Persisting in Saliva for More Than 15 Months After Infection and Become Strongly Boosted After Vaccination”, my contribution (estimation in %) was as follows:

- Experiments were planned with Rolf Fendel, and ELISA measurements were predominately performed by me.
- Data analysis and verification was conducted with the following contributing co-authors: Yudi T. Pinilla, Douglas Consolaro, Rolf Fendel, Jana Held, and Andrea Kreidenweiss.
- The manuscript draft was written with the co-authors Andrea Kreidenweiss, Yudi T. Pinilla and Rolf Fendel.

For the following publication, “Saliva and Plasma Antibody Levels in Children and Adolescents After Primary Infection With Omicron Variants of SARS-CoV-2 Infection in Germany”, my contributions were in the following sections:

- The study's concept and design were drafted with the following co-authors: Peter Kremsner, Jana Held, Rolf Fendel, and Andrea Kreidenweiss.
- Acquisition, analysis, and interpretation of data.
  This work was accomplished with Yudi T. Pinilla, Douglas Consolaro, Ayla Binder, Vanessa Krohmer, Jana Held, Rolf Fendel and Andrea Kreidenweiss.
- The drafting of the manuscript was achieved in collaboration with Yudi T. Pinilla, Rolf Fendel and Andrea Kreidenweiss.

# 1. Introduction

## 1.1. SARS-CoV-2 virus origin and characteristics

In December 2019, the first cases of the disease COVID-19 (Coronavirus disease 2019) were reported in Wuhan, China, caused by a virus later known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1,2). Since its emergence, the rapid transmission of the SARS-CoV-2 virus has led to a global pandemic, fundamentally disrupting human society (1,2). As of 7$^{th}$ January 2024, the WHO has reported 774 million confirmed cases and estimates more than seven million fatalities due to COVID-19 (3).

The origin of SARS-CoV-2 remains unclear. The most likely scenario discussed is a zoonotic spillover event, but a laboratory leak cannot be excluded completely (4,5). SARS-CoV-2 positive environmental samples from the Huanan market with wild animals and the geographical proximity of the market to the first human SARS-CoV-2 infections support the hypothesis of a zoonotic event (4,5). The exact chain of transmission from animal to human has not been identified, with wild bats being a likely reservoir (2,5). SARS-CoV-2 is an enveloped, positive, single-stranded RNA virus that belongs to the family of *Coronaviridae* and the genus of Betacoronaviruses (1,6). Other related Betacoronaviruses that can infect humans are the SARS-CoV-1, which was contained after its outbreak in 2002/2003, and the MERS-CoV virus (Middle East Respiratory Syndrome Coronavirus) (1,6,7). A distinct characteristic of SARS-CoV-2 compared to SARS-CoV-1 is the composition of the Spike protein (S-protein), especially the binding region at the tip that is composed of the receptor binding domain (RBD) (8). The RBD region mediates the entry of the virus into the human host cell by binding to the angiotensin-converting enzyme 2 (ACE2) receptor (8,9). Due to mutations at the RBD region, the SARS-CoV-2 virus efficiently infects epithelial cells in the human respiratory tract and quickly spreads in the naïve population (8).

## 1.2. SARS-CoV-2 pandemic and variants of concern (VOC)

The SARS-CoV-2 virus rapidly expanded from China to the rest of the world and was declared by the WHO on 11th March 2020 as a global pandemic (10). In response, many countries implemented travel restrictions and lockdown measures in 2020 to slow the spread of the virus (11). During the pandemic, various strategies were applied

to mitigate the transmission of the virus, including social distancing, personal protective measures like mask-wearing, temporary school closures, and widespread diagnostic testing to reduce the burden on healthcare systems (12). The gold standard for detecting acute SARS-CoV-2 infection involves the amplification of viral RNA by methods such as real-time reverse transcription-polymerase chain reaction (RT-qPCR) (12). A turning point during the pandemic was the exceedingly fast development of novel and effective vaccines against SARS-CoV-2, with the first one hitting the market in December 2020 (13,14). Different types of vaccines were approved, including highly effective mRNA-based formulations from Pfizer-BioNTech and Moderna (13).

During the pandemic, new variants of the SARS-CoV-2 virus appeared with increased infectious properties (11). While spreading, the RBD and S-protein of SARS-CoV-2 underwent additional mutations, enhancing its adaptation to the human host and increasing virus transmission (15). Highly mutated variants of SARS-CoV-2 emerged, and they were classified by the WHO as variants of concern (VOC) for additional observation (15). The most well-known VOCs include the Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), Gamma (P.1), and Omicron (B.1.1.529) strains (15). These variants emerged sequentially and often replaced previous variants due to enhanced viral properties (15,16).

The first VOC, Alpha (B.1.17), was identified in September 2020 in the United Kingdom (11). The Alpha variant exhibited a stronger binding to the ACE2 receptor due to a point mutation of asparagine to tyrosine in the RBD region (11). This enhanced affinity for ACE2 led to an estimated 50% increase in transmission compared to previous strains, facilitating its rapid global spread (17,18). Subsequently, two additional VOC strains, Beta (B.1.351) and Gamma (P1), emerged in South Africa and Brazil. Like Alpha, these variants displayed mutations in the Spike protein, enhancing their binding to the ACE2 receptor while potentially reducing the binding of neutralizing antibodies (11,19).

In mid-2021, the Delta variant quickly rose to dominance globally, with an estimated increase in transmissibility of 55% (based on WHO data from the UK and India) compared to the Alpha strain (20,21). Delta displayed new mutations at the RBD region of the spike protein (L452R and P681H) close to the furin cleavage site, resulting in increased replication, producing 6 times more viral RNA copies than Alpha (15,20). Delta spread rapidly in the population, giving it a competitive advantage over previous variants (21). The estimated effective reproduction number, which represents the

expected number of infections caused by acutely infected individuals in a population with some individuals not being susceptible, showed a 97% increase in Delta compared to 55% in Alpha, 60% in Beta, and 34% in Gamma compared to non-VOC/VOI (variants of interest) strains (21). Notably, Alpha, Beta, Gamma, and Delta have been reported to have increased hospitalization, ICU ventilation, and mortality rates compared to the wild-type virus, with Delta and Beta posing a higher risk than Alpha and Gamma variants (22).

At the end of 2021, the emerging Omicron strain (B.1.1.529) swiftly replaced Delta worldwide (23). Compared to previous strains, Omicron is currently the most immune-evasive and transmissible SARS-CoV-2 variant, being approximately 3.2 times more transmissible than Delta (23). Omicron was first discovered in Botswana and South Africa and was officially reported by the WHO in November 2021 (24). One hypothesis to explain the high mutation rate in this variant is an accelerated evolution of the virus within the immunodeficient population, including individuals with conditions such as HIV, who may harbour persistent, non-lethal SARS-CoV-2 infections, with long-term pressure between the host immune system and the virus adaptations (24). Other potential explanations include silent mutation occurring within populations with limited or no surveillance via sequencing or evolutionary changes in another species, such as rodents (23,24). The Omicron variant quickly evolved into multiple lineages like BA.1, BA.1.1, BA.2, BA.3, BA.4 and BA.5 (24).

Omicron drastically changed the course of the pandemic due to its high level of immune evasion and resistance to neutralizing antibodies from convalescent and vaccinated individuals (23). However, the pathogenicity of Omicron in adults was substantially reduced, with less severe cases and hospitalizations (23). The Omicron spike protein contains about 30 mutations, with 15 located in the RBD, possibly interfering in the recognition and binding of RBD as the central target for neutralizing antibodies (25). Additionally, the S1 unit contains an N-terminal domain (NTD), another antigenic site, that is blocked from antibodies due to new mutations (25). Other viral proteins like the envelope (E), nucleus (N), and membrane (M) proteins might have additional mutations which enhance virus replication, transmission, and host immune evasion (25). Interestingly, the binding ability of Omicron RBD to the ACE2 is comparable to or weaker than the previous Delta variant and much weaker than the Alpha variant, supporting that other factors drive the enhanced transmission and infectiousness of Omicron (23). In summary, the emergence of Omicron made reinfections with

SARS-CoV-2 much more common in vaccinated and previously infected individuals (24). Notably, vaccinations substantially reduce the risk of hospitalization and severe infection, making it a crucial factor in the control efforts against SARS-CoV-2 (24).

New Omicron variants continue to emerge with high immune evasive properties. However, global fatalities and hospitalizations have declined due to lower symptomatology associated with Omicron and widespread population immunity through previous exposure and vaccinations against SARS-CoV-2 (26). In May 2023, the WHO announced the end of the public health emergency of international concern related to SARS-CoV-2 (26).

### 1.2.1. SARS-CoV-2 pandemic in Germany

The SARS-CoV-2 pandemic in Germany exhibited a transmission pattern characterized by periods of acceleration and deceleration (27). This characteristic generated waves in the pandemic, with a significant increase in newly infected cases at the beginning, followed by peak and subsequent deceleration (27). The intensity of these pandemic waves changed over time. Influencing factors include different virus variants with varying transmission rates and symptomatology, seasonal effects, public health control measures and the level of previously acquired immunity in the general population (27).

In early 2020, SARS-CoV-2 infections emerged in Europe, with the first cases in Germany reported on January 28th (28). The country faced its first wave from April 2020 to July 2020, with over 190,000 confirmed infections (29,30). About 18% of the SARS-CoV-2 confirmed cases were hospitalized, with 14% being in the intensive care unit, consisting of mostly older patients (30). In the age group of 80 years and older, every second COVID-19 infection led to hospitalization and every third case to death (30). Independent of hospitalization and symptomatology, about 5.6% (8616 out of 152,984 cases) of the patients included in the RKI report died (30). However, approximately 80% of SARS-CoV-2 infections were uncomplicated, with seroprevalence studies suggesting that 27 to 40% of post-outbreak cases were asymptomatic (30,31). Information regarding SARS-CoV-2's impact on children remained scarce, particularly in the early days of the pandemic (see section 1.2.2.).

In Germany, the second wave of SARS-CoV-2 quickly accelerated in autumn 2020 and lasted until February 2021, with a peak in December 2020 (27,29). Over 2 million

SARS-CoV-2 cases were reported in Germany, with similar characteristics in the hospitalized population compared to the first wave (29). On the 27th of December 2020, the vaccination program in Germany started, prioritizing healthcare workers and elderly individuals (29). During the third wave in Germany from February 2021 to June 2021, with about 1.4 million infections, the newly emerged Alpha variant (B.1.1.7) heavily influenced the course of the pandemic with its increased virulence (27,29). In April 2021, about 91% of the reported SARS-CoV-2 cases in Germany were from the Alpha variant (32). During the third wave, COVID-19 outbreaks occurred less often in hospitals and long-term care facilities compared to previous waves, likely due to the increase in number of vaccinated individuals (29).

Starting in July 2021, the Delta strain quickly became dominant in Germany and globally, resulting in a 4th infection wave (33). Vaccination efforts showed excellent efficacy against severe COVID-19 cases and fatalities, with approximately 65% of the population receiving vaccination by August 2021 (33). By the end of 2021, Delta-driven transmissions urged to unprecedented levels in Germany, reaching a new daily case count of about 50.000 on December 15th, which was exceeded only by the Omicron-induced infections a few weeks later, with nearly 295.000 new cases on March 18th (33,34). The newly emerging Omicron variant quickly replaced the Delta variant and became dominant from the 2nd week of January 2022 onwards, with new subvariants (BA.2; BA.4, BA.5, etc.) of Omicron appearing in the following weeks (35). On November 25th, 2021, the European Medicines Agency (EMA) approved a vaccine for children aged 5 and 12 (36). Compared to the Delta variant, Omicron exhibited increased transmission, reduced disease severity, and notably lower rates of intensive care admissions in German hospitals, with a higher proportion of young patients (37,38).

### 1.2.2. SARS-CoV-2 infection and seroprevalence in children and adolescents in Germany

Early in the pandemic, information on children, especially younger children and infants, was limited, and their role in transmission in the pandemic was highly debated (39). In the first two years of the pandemic, many schools and preschool facilities were temporarily closed (lockdown) or restricted by reducing the number of children and implementing hygiene routines to prevent and reduce virus transmission (39,40).

The first studies from 2020 found that the infection rate among 0- to 9-year-old children was the lowest compared to the other age groups in Germany, Italy, South Korea, and China (31). In a study by Loss et al., young children were monitored in daycare facilities and households from autumn 2020 to summer 2021, revealing that exposed adult employees had a higher risk of contracting SARS-CoV-2 than preschoolers (40). In general, a very heterogenous transmission risk was observed in daycare facilities, with an overall higher risk of transmission in the respective households (40).

In Bavaria, nearly 60,000 children and adolescents were recruited for a SARS-CoV-2 seroprevalence study as part of a screening program for presymptomatic type 1 diabetes from January 2020 to June 2022 (41). Results from June 2022 show an overall seroprevalence rate in children of 73.5%, with preschoolers exhibiting a lower prevalence (67%) compared to school children (84%; 5 – 10 years old) (41). During this period, 78% of the children in Germany aged 5 – 10 years and 28% of adolescents (12 – 17 years) did not receive their first vaccination (41). This indicates, with the prevalence data, that a significant proportion of children were infected by SARS-CoV-2 during the Omicron wave. Before the emergence of Omicron, in November 2021, the total seroprevalence was only at 14.7%, with 16.2% of school children and 13% of preschoolers (41). In summary, a substantial increase in infections among children and adolescents was observed in Germany with the emergence of Omicron variants (41).

### 1.2.3. SARS-CoV-2 house-hold transmission in children

In the early stages of the pandemic, children did not play a significant role in household transmission, but with the emergence of VOC strains and much later initiation of vaccination programs for children, the question of disease transmission became quite complex (42,43). Overall, vaccination appears to be a major tool in reducing the risk of SARS-CoV-2 transmission within households (43). Studies have found that the viral load of asymptomatic and mildly symptomatic COVID-19 cases in children is comparable to or slightly lower than that in adults with similar symptoms (44,45). Children and young adults (< 20 years) were reported to have lower odds of experiencing a secondary SARS-CoV-2 infection compared to older adults (46,47).

## 1.3. COVID-19 symptomatology in adults and children.

SARS-CoV-2 causes respiratory infections in humans that can be asymptomatic or manifest in mild to severe symptoms (4). The most commonly reported symptoms of COVID-19 are dry cough, headache, fatigue, fever, and, in more severe cases, acute respiratory distress syndrome (ARDS), viral pneumonia, and dyspnoea (1,4). Other symptoms include olfactory dysfunction (such as loss of smell and taste, although less commonly reported with Omicron), gastrointestinal symptoms, and cardiovascular complications (48,49).

The immune system of most patients can clear the infection without complications, but with age and pre-existing health conditions, the risk of severe COVID-19 substantially increases (48,50). As reported by the Centers for Disease Control and Prevention (CDC) in the USA, 90% of deaths occurred in adults older than 50 years (50,51). Other risk factors include lack of vaccination, comorbidities like diabetes, obesity, and hypertension, and the type of the SARS-CoV-2 variant contracted (22,52). Compared with adults, children rarely develop severe COVID-19, which usually occurs in combination with comorbidities (53). In a COVID-19 study conducted in 2020 in China by Wu et al., with more than 72,000 COVID-19 cases, children accounted for only 1% to 2% of hospitalizations (53,54). Especially early in the pandemic, most children and adolescents were asymptomatic or had mild COVID-19 and, therefore, were often not detected and underrepresented in SARS-CoV-2 studies (50).

Different variants of SARS-CoV-2 seem to induce various symptoms in children. In a study with 1440 participants in Canada, children with the Delta and Omicron variants had more symptoms, especially fever, than those infected by the Alpha strain (55). In this study, the number of children admitted for hospitalization did not change between variants (55). Contrary to children, adults with Omicron displayed reduced symptomatology and a shortened duration of sickness (49).

Apart from the symptoms of an acute COVID-19 infection, children and adolescents can experience severe aftereffects, including inflammatory manifestations such as multisystem inflammatory syndrome in children (MIS-C) and myocarditis (56). Long-term sequelae following COVID-19 are increasingly recognized in the form of long COVID or post-COVID syndrome, which can affect both children and adults, with persistent symptoms like fatigue, cognitive impairment, and dyspnoea (56). Underlying mechanisms likely include immune dysregulation, autoimmunity, or potentially viral

persistence (57). In summary, while long COVID is less often in children, the long-term effects and underlying mechanisms remain unclear (57).

## 1.4. SARS-CoV-2 infection and immune response

The infection process of SARS-CoV-2 begins with the entry of viral particles via the upper respiratory tract, aiming to infect human cells and replicate fast (58). If the host immune system does not stop the SARS-CoV-2 virion, it attaches to a cell by using the S1 unit of the spike protein to bind the ACE2 receptor on the cell surface (59). A furin cleavage motif between the S1 and the S2 unit is targeted by the host transmembrane serine protease TMPRSS2, separating both spike units (59). Subsequently, a fusion with the cell membrane is mediated by the S2 domain of the spike protein, allowing the release of the viral genomic RNA into the host cell (59). After cell entry, SARS-CoV-2 initiates the production of viral proteins for replication at the endoplasmic reticulum to repeat the infection cycle (59).

The entry point of the SARS-CoV-2 virus, the respiratory tract, is guarded by mucosal and innate immunity, which involve membrane – and cytosolic receptors (58). These can activate a potent anti-viral type-I-interferon response, with subsequent antigen-mediated activation of the adaptive immune response (58). The innate and adaptive immune system fights the viral intruder by releasing inflammatory cytokines and activating immune cells, such as cytotoxic T-cells, to eliminate virus-infected cells (48). Antigen-presenting cells like dendritic cells mediate T helper cell (CD4+) and B cell activation and differentiation to combat the viral infection (58). In the later phase of the immune response, large quantities of antibodies are secreted by plasma cells to neutralize the virus by blocking the binding domain of the spike protein and, therefore, inhibiting the ability of the virus to infect cells (58). In previously naïve patients, SARS-CoV-2 infection induces after 10 days (range 5 to 14 days) IgM, IgA, and IgG antibodies, with a significant correlation between SARS-CoV-2 specific IgG and neutralizing antibodies (60). The IgG antibody response reaches its peak in the third week post-symptom onset, with much higher IgG titers observed in hospitalized patients (60). In cases with severe COVID-19, an exuberant immune response has been observed, with high cytokine levels (cytokine storm) and lower CD4+ and CD8+ cells leading to immune dysregulation (61).

### 1.4.1. Humoral response after SARS-CoV-2 vaccination

The humoral response following SARS-CoV-2 vaccination is heavily influenced by multiple factors: the type of vaccination administered, previous vaccinations and infections, and the overall immune response of the individual (60,62). Available vaccines include nucleic acid vaccines, such as mRNA vaccines (vaccines by Pfizer–BioNTech and Moderna), vector vaccines (vaccine by AstraZeneca, Sputnik-V), protein-based vaccines containing SARS-CoV-2 proteins (Novavax), virus-like particles, as well as live-attenuated or inactivated virus (SinoVac) (63). Most vaccines target the Spike protein, specifically the RBD, as it is the main neutralizing structure of SARS-CoV-2 (63).

The widely used and highly effective BNT162b2 vaccine from Pfizer–BioNTech induces a strong humoral immune response in serum and saliva, especially after the second dose (62). Individuals who received two doses of the vaccine and those who recovered from a natural infection show similarly high levels of IgG targeting RBD (62). However, NCP-specific antibodies are only found in the previously infected group, as NCP antibodies are not induced by the vaccine (62). Notably, the humoral response is significantly lower in participants aged 60 and older and in individuals with conditions such as diabetes and cardiovascular morbidities (64). Overall, receiving three vaccinations enhances the antibody response, extends the duration of antibody half-life, and helps induce an adequate immune response, particularly in elderly and immune-deficient populations (64,65). The antibody response after three doses of BNT162b2 peaks 24 days after the injection and has an estimated half-life of 80 days (46–303 days) (65).

### 1.4.2. Role of the mucosal immunity at the respiratory tract

Mucosal immunity provides a first line of defence against invading microorganisms and viruses at entry points like the respiratory system (66). The upper respiratory tract is constantly exposed to the outside world through the uptake of oxygen and nutrients and is colonized by resident or opportunistic microbiota (66). The mucosal site provides a physical (epithelial cells) and an immunological barrier (antibodies, peptides, etc.) to protect the host (66). Through airborne droplets containing viral particles, the SARS-CoV-2 virus primarily infects the upper respiratory tract and its mucosal surfaces (67). SARS-CoV-2 infection in the upper respiratory tract is associated with mild

symptoms, but when reaching the lower respiratory tract, SARS-CoV-2 causes severe symptoms like pneumonia (67).

With previous exposure to the virus or vaccination, the saliva is enriched with SARS-CoV-2 binding antibodies to protect mucosal surfaces by inhibiting the attachment of the virus to the host cells (68). Salivary IgG derives from the plasma and is passively transported by transmucosal diffusion into the oral cavities (69). The IgG levels in saliva and plasma correlate well, and salivary IgG can persist over many months (70). Another cornerstone of humoral immunity is the secretory IgA (sIgA), which is produced by plasma cells in the lamina propria of the mucosae (71). While IgA in serum is monomeric, in saliva and other secretions, IgA predominately exists in a dimeric form (68). For enhanced stability and protection from degradation, both IgA components are linked with a glycoprotein called J-chain and a secretory component (71). In saliva, sIgA is crucial in inhibiting infection processes of bacteria and viruses by blocking pathogen surfaces (71). Research on the influenza virus shows that virus neutralization by virus-specific sIgA is even more effective than IgG (72). In general, the humoral response of IgM and IgA is induced earlier than IgG, but they have a much shorter half-life (70). For SARS-CoV-2 infections, depending on the severity of the symptoms, sIgA quickly declines after 6 to 10 weeks after infection (73).

## 1.5. Saliva and its role as a diagnostic fluid

In the past decade, saliva has emerged as a valuable diagnostic tool due to its rich content of biomarkers, proteins, and genetic material (74). Saliva specimens have been utilized to detect and monitor diseases like SARS-CoV-2, HIV, diabetes, and autoimmune and periodontal diseases (74,75). Many biomarkers in the body and blood are reflected well in saliva through active transport or passive transduction via the gums, such as IgG antibodies (69,74). In studies, saliva collection is non-invasive and easily scalable (75). Saliva can be sampled directly by the patient, and no medical staff is required for venipuncture procedures (75). This pain-free, non-invasive collection approach is especially beneficial when working with vulnerable populations such as children (74). Although concerns have previously been raised regarding low concentrations of target molecules in saliva, advancements in molecular techniques have greatly enhanced sensitivity, making saliva an increasingly viable alternative to blood (74). Protocol procedures require a specific adaptation when working with saliva to gain high-sensitive detection and robust quality results (75).

The optimization of an enzyme-linked immunosorbent assay (ELISA) can be extensive, as every step of the assay can be improved to enhance accuracy, robustness, sensitivity, and specificity (76). A cohort of confirmed positive and negative samples can be utilized to find optimal assay conditions and components to minimize cross-reactivity and background signals and maximize sensitivity and specificity (76). Repetitions of the assays, including inter- and intra-assays, are crucial for ensuring reproducible results and assessing the precision of the test (76). Especially for saliva samples, the methodology of sample collection, handling, and stability requires further investigation (74,76).

## 1.6. Epidemiological study to assess the prevalence of SARS-CoV-2 infection in children and adolescents in Tübingen, Germany

A longitudinal, observational, prospective study to assess the prevalence of SARS-COV-2 infection in children and adolescents living in the city of Tübingen, Germany (acronym Coro-Buddy) was initiated in early 2020 (77). The special feature of this study was the measurement of antibodies in saliva. The hypothesis was that SARS-CoV-2 infections in the children and adolescent cohorts might be underestimated by active viral detection tests (first by molecular diagnostics and later by antigen testing) due to limitations of diagnostic tests, symptom-based testing strategy, possible asymptomatic infection of children and youths, etc..

Saliva samples were collected longitudinally from participants to detect SARS-CoV-2 RBD-specific IgG in individuals. Educational facilities like pre-, primary and secondary schools were approached for study participation. In total, 2093 children and adolescents aged 1 to 18 were included in the study for a longitudinal follow-up (unpublished data). Approximately 15% of the children in Tübingen participated in the study, forming a close-to-representative cohort of the administrative city of Tübingen. Four collections were conducted for July 2020 (n = 749, included participants), October to December 2020 (n = 1699), April to July 2021 (n = 1498) and March to May 2022 (n = 1058) (unpublished data). A questionnaire was handed out to collect information about recent cold-like symptoms, positive SARS-CoV-2 tests, SARS-CoV-2 infections in the household and the SARS-CoV-2 vaccination status.

The study further included adults (n = 72) who were well-defined for their infection status and concomitant saliva and plasma sampling. During the 15-month follow-up,

58% of the adults received the first COVID-19 vaccination, predominantly mRNA-based. RBD-IgG levels and neutralizing activities were compared to non-vaccinees.

# 2. Objective of the thesis

The role of children at the beginning of the COVID-19 pandemic was a subject of extensive discussion due to the limited information on population exposure to this novel disease (39,78). Therefore, serological IgG antibodies specific to SARS-CoV-2 were investigated by seroprevalence studies as a marker of prior infection (79). Typically, these studies assess antibodies in serum samples. However, sampling of salivary specimens presents a non-invasive alternative that can be conducted in non-medical settings (75). This method is well-tolerated by children and allows mass screening (74,75). Therefore, this project aimed to:

1. Develop a robust and specific saliva-based ELISA assay for detecting SARS-CoV-2 RBD-specific IgG.
2. Apply this methodology in a population-based, cross-sectional, longitudinal, and prospective prevalence study with children from Tübingen (Coro-Buddy) during the COVID-19 pandemic (2020 - 2022, including four collection time points).
3. Compare the SARS-CoV-2 antibody prevalence and immune response of different age groups and the influence of outbreak waves on population exposure.
4. Determine the salivary antibody response and persistence in vaccinated and convalescent participants.
5. Investigate the impact of SARS-CoV-2 variants of concern (VOC), emphasising primary Omicron convalescence and how it compares to previous variants in unvaccinated youths.

# 3. Results

The results of this study are summarized in three publications:

## 3.1. Non-Invasive Antibody Assessment in Saliva to Determine SARS-CoV-2 Exposure in Children

For a non-invasive detection of the SARS-CoV-2 exposure and humoral antibody response, a saliva-based ELISA for SARS-CoV-2 RBD-specific IgG was developed. To characterize the performance of this method, paired plasma and saliva samples from adults were used. Included were convalescent individuals with a previous SARS-CoV-2 test (n = 75), participants with no positive test (n = 108), and pre-pandemic samples (n = 32, only plasma). The specific IgG levels were characterized using two certified ELISA assays (Euroimmune and Kantaro COVID/SeroIndex). Subsequently, 47 convalescent and 99 negative samples with verified IgG status in plasma were tested with the new saliva ELISA protocol. A ROC curve was generated, with a maximized specificity of 100% (95% CI 96% - 100%) and a sensitivity of 87% (95% CI 75% - 94%). Assay performance was assessed via an intra- and inter-assay, with the coefficient of variation for the former well below the cut-off of 10% even in strong dilutions and 3% to 13%, respectively (sample dependent). Saliva specimens were tested for stability, revealing rapid degradation at room temperature but stable behaviour at 4°C and during multiple freeze-thaw procedures.

The saliva-based ELISA assay was implemented in the Coro-Buddy study, a longitudinal, observational, and prospective study conducted in the Tübingen city district. The study aimed to assess SARS-CoV-2 exposure and antibody response in preschool and school environments. In 2020, 1850 children and adolescents were recruited during the first two collection periods. About 12% of all children in Tübingen aged 1 to 10 years participated (n = 837 participants). The total SARS-CoV-2 RBD IgG prevalence was 1.6%, indicating a 3 times higher exposure in children than PCR data from the health department showed. Interestingly, the prevalence in primary school children (6-10 years; n = 333) was at 3.0%, significantly higher (p = 0.0047, Fisher's exact t-test) than the prevalence of 0.4% in preschool children (1-6 years; n = 504). In total, 12 children had a positive IgG status, with three individuals potentially exposed within their households, and only one participant was aware of a past SARS-CoV-2 infection. Overall, 84% of the positive cases (10/12) were asymptomatic.

## 3.2. SARS-CoV-2 Antibodies Are Persisting in Saliva for More Than 15 Months After Infection and Become Strongly Boosted After Vaccination

The available information on antibody persistence and kinetics after SARS-CoV-2 infection and vaccination was incomplete, especially during the first year of the pandemic. To address this knowledge gap, 72 COVID-19 convalescent individuals were followed up for up to 15 months. These participants were diagnosed with COVID-19 from May to October 2020 and had experienced mild symptoms. No reinfections were reported during the study. The initial collection (only plasma) was performed 4 months post symptom onset (PSO), showing that 89% (64/72) of participants were positive for RBD-specific IgG in the in-house ELISA. These results were confirmed using a commercial ELISA test (Euroimmune) with 81% (58/72) IgG positivity for the spike protein and 5 intermediate results. Six months after infection, IgG positivity in plasma remained at 89% and reached 72% (46/72) in saliva.

At the end of the follow-up at the 15-month endpoint or the last collection before vaccination, ELISA results showed that 98% (63/64) of participants were IgG-positive in plasma and 80% (37/46) in saliva. The IgG concentration remained constant, displaying flat regression lines. A strong correlation between IgG levels in plasma and saliva was observed, with a Pearson correlation of $r = 0.7$ (95% CI 0.67 – 0.78). Overall, the median IgG concentration in saliva was 1206-fold lower compared to plasma.

Between 7 to 14 months post-infection, 58% of the participants (42/72) were vaccinated against SARS-CoV-2 for the first time. Most received mRNA-based vaccines (29/43), followed by vaccines from AstraZeneca (13/43). One month after vaccination, a 72-fold increase in RBD-specific IgG in plasma and a 46-fold increase in saliva were observed, a significant rise compared to non-vaccinated individuals ($p<0.001$). Interestingly, previously IgG-negative individuals had 3.3-fold (plasma) and 4.6-fold (saliva) lower IgG levels one month post-vaccination. In addition, an ACE2 receptor antibody competition assay (NeutraLISA, Euroimmune) was performed with plasma to serve as a surrogate for virus neutralization. Four months after infection, 34% (17/50) of convalescent individuals showed neutralizing activity, which declined to 27% (14/50) at 8.5 months PSO. A significantly higher inhibition rate ($p<0.001$) was detected in participants who received the first vaccination dose two months earlier, with a 100% (33/33) inhibition rate. A distinct correlation between the IgG concentration and neutralizing activity was observed ($r = 0.7$).

## 3.3. Saliva and Plasma Antibody Levels in Children and Adolescents After Primary Infection With Omicron Variants of SARS-CoV-2 Infection in Germany

By the end of 2021, the rapid spread of the new Omicron variant fundamentally changed the course of the pandemic, with the strain displaying strong immune evasive properties. While most adults were already vaccinated, many younger children had not yet received vaccination. Only scant information was available on antibody prevalence after primary Omicron infection, especially for salivary antibodies in youths.

During the Coro-Buddy study, participants aged 1 to 18 were recruited for saliva collections in spring 2021 (n = 1474) and spring 2022 (n = 1194). Individuals who reported a previous SARS-CoV-2 infection confirmed by PCR or antigen test between April 2020 and early December 2022 (n = 54) were classified as “pre-Omicron”. Participants with an infection between the second week of January 2022 and May 2022 (n = 173) were categorized as the “Omicron” group based on the predominant Omicron variant (BA.1 and BA.2) during that period (RKI weekly reports). The average time between infection and sample collection was 175 days for the pre-Omicron group and 53 days for the Omicron group.

The significantly lower IgG positivity specific for RBD WT (wild type or ancestral strain in the Omicron group (32/173), compared to the pre-Omicron group (37/54) was remarkable ($p < 0.001$ by Wilcoxon rank sum test). Other antigens were tested to validate this observation, revealing similarly low IgG positivity in the Omicron group: RBD-BA.1 (14/64), RBD-BA.2 (9/64), Spike Trimer (Tri-BA.1) (16/51) and NCP-BA.1 (14/64). Individuals with two SARS-CoV-2 infections (pre-Omicron/ Omicron or two Omicron infections, n = 13) showed an enhanced antibody response in saliva compared to those with a single Omicron infection. Vaccinees with at least two doses of mRNA vaccines exhibited the highest IgG levels in saliva among youths, with 99.7% IgG positivity for RBD WT (324/325).

A subset of convalescent participants was invited for an additional collection of blood and saliva for further investigations. Individuals from the pre-Omicron group displayed high IgG concentrations with 100% IgG positivity in plasma against RBD WT and Spike Trimer WT. In contrast, Omicron convalescent participants had IgG levels close to the cut-off, with IgG positivity in the Omicron group ranging from 47% (8/17) for RBD-WT, 65% and 71% for RBD-BA.1 and BA.2, and 67% for NCP-BA.1 (10/15). In summary,

plasma measurements reflected the low IgG levels observed in saliva after primary Omicron infection in children.

# 4. Discussion

## 4.1. Non-Invasive Antibody Assessment in Saliva to Determine SARS-CoV-2 Exposure in Young Children

### 4.1.1. Saliva RBD-specific IgG ELISA assay establishment

Antibodies are a cornerstone of the humoral immune system, playing a critical role in defending against viral infections like SARS-CoV-2. Particularly in the upper respiratory tract, mucosal antibodies provide the first line of protection against viral invaders (66). Following infection, the presence of antibodies can be analyzed to detect previous exposure/infection to SARS-CoV-2. Notably, several reports have shown that children and adolescents often experience asymptomatic SARS-CoV-2 infections (50,80), making it difficult to measure exposure without assessing the immune response.

In the first year of the pandemic, children were an underrepresented population group in research, with limited data on prevalence and antibody response to SARS-CoV-2 (39,80). This project aimed to address this gap by establishing a non-invasive collection strategy, using saliva specimens to encourage participation from young children. Since no commercial ELISA for analyzing saliva was available in 2020, an in-house ELISA was developed targeting salivary RBD-specific IgG in saliva. Previous publications by Kim et al. (1992) and Isho et al. (2020) reported that IgG from plasma transudates into saliva and that plasma IgG correlates well with IgG levels in saliva (69,70). This observation was used for the ELISA establishment by collecting paired saliva and plasma samples from SARS-CoV-2 convalescent (n = 47) and negative individuals (n = 99). Plasma samples from participants with and without a positive SARS-CoV-2 test were confirmed for SARS-CoV-2 specific IgG with certified ELISA Kits (Euroimmune and Kantaro) before being selected in the saliva establishment cohort.

During the first year of the pandemic, the overall prevalence of SARS-CoV-2 was low (81–83), requiring a high specificity and accuracy of the test. Our assay was optimized for a sensitivity of 87% (95%CI: 75% - 94%) and 100% specificity (95%CI: 96% - 100%) based on ROC curve analysis. For comparison, a salivary IgG RBD

assay developed by MacMullan et al. (2020) reported a sensitivity of 84% and specificity of 100% across 149 clinical samples (84). However, their protocol included an additional step where the saliva IgG samples were concentrated beforehand by an Amicon filtration and centrifugation, making the sample preparation process more complicated and expensive and reduces throughput. In comparison, Chiang et al. (2021) developed an electrochemical assay for the detection of salivary S1-specific IgG, which achieved a sensitivity of 88% at a maximized specificity of >99.9% (85). Another study by Constantini et al. (2022) developed a saliva-based IgG assay against the spike protein, with 187 PCR-confirmed cases and 373 pre-pandemic saliva samples, resulting in an assay sensitivity of 89.7% and a specificity of 97% (73). For reference, the Euroimmun kit was one of the first widely used commercial ELISA for detecting S1-specific IgG in serum and plasma, showed a sensitivity of 89.5% (75.3 – 96.4) and a specificity of 96.1% (90.1 – 98.8) according to Van Elslande et al. (2020) (86). In summary, other saliva-based SARS-CoV-2 specific IgG EIAs (enzyme immune assays) and serum-based commercial ELISA kits displayed comparable specificity and sensitivity to the in-house saliva ELISA.

For the validation of the ELISA, inter- and intra-assay variability tests were performed to assess the stability and accuracy of the test with samples ranging from high to low IgG values. In the inter-assay, the resulting variability was below the 15% precision cut-off (3 – 13 % range). In the intra-assay, measurements were well below the precision cut-off of 10% (1 - 8% range), especially in the first dilution steps. The values for the precision cut-offs were oriented on a salivary ELISA validation for cortisol by Thomson et al. (2014) (87). In the salivary electrochemical assay described by Chiang et al. (2021), an inter- and intra-assay variability of 16.2 and 9.3% was stated (85). In the publication of Martínez-Subiela (2022), the salivary SARS-CoV-2 immunoassay against RBD-specific IgG resulted in an inter- and intra-assay CV range between 13 – 15% and 6 - 13% respectively (88). Our in-house assay demonstrated similar or lower variability than the above-stated assays for salivary RBD-specific IgG.

In general, the performance and sensitivity of an assay can highly depend on the components used, including antigens, detection antibodies, and buffers. In a study by Pisanic et al. (2021), eleven SARS-CoV-2 antigens from different suppliers or origins (4 RBD antigens) were tested for accuracy (89). The RBD antigen (Mt. Sinai, used in the in-house ELISA) had the highest specificity and sensitivity, together with another RBD antigen (GenScript N), when measuring IgG in both saliva and plasma (89).

Another factor to consider is the disease severity of the recovered participants used for the assay establishment. Asymptomatic patients generally induce a much lower IgG response than severely ill SARS-CoV-2 patients (85). In the Coro-Buddy study, none of the participants were hospitalized, and all exhibited mild symptoms, resulting in overall moderate to low antibody levels. Which resembles more closely the mild symptoms often seen in children and adolescents, the target group of the study (90).

Regarding the establishment of the saliva-based ELISA, it is important to acknowledge certain limitations: the assay optimization was performed on saliva from adults and then applied to youths without further adjustments for potential age-related differences in antibody levels (further discussed in section 4.1.2.). Another limitation was the absence of pre-pandemic saliva samples that could serve as an additional negative control group. Unlike blood, saliva was less commonly sampled and not readily available. The negative cohort included healthy individuals reporting no SARS-CoV-2-related symptoms and without a positive SARS-CoV-2 test. Furthermore, all participants were tested negative for RBD-specific IgG in plasma. In addition, the low SARS-CoV-2 prevalence in early 2020 posed a minimal risk of a prior unrecognized infection (82,83).

During assay establishment, potential cross-reactivity was not examined. Cross-reactivity might be associated with other beta coronavirus strains (SARS-CoV, MERS-CoV), endemic coronaviruses (HCoV-OC43 and HCoV-HKU1) and other respiratory diseases like influenza. Notably, SARS-CoV-1 and MERS-CoV have almost no recorded cases in Europe, making it extremely unlikely to encounter them (91). The endemic beta coronaviruses, which can cause what is commonly known as a cold, are widespread in the population. Considering the high prevalence and reinfection rate (92), acquiring a verified cohort of negative individuals may be challenging. Nevertheless, cross-reactivity induced by endemic strains might be partially represented in a populous negative cohort.

In summary, a functional ELISA for detecting salivary RBD-specific IgG was successfully established, including the in-house production of the RBD antigen. The in-house assay demonstrated high sensitivity and specificity with below-average inter-assay variability compared to other published highly sensitive assays. Limitations of the assay optimization included the absence of pre-pandemic saliva samples and the lack of cross-reactivity testing for other Coronaviruses.

### 4.1.2. SARS-CoV-2 prevalence data based on salivary IgG from young children in Tübingen city in 2020

The optimized ELISA for detecting salivary, RBD-specific IgG was implemented during the Coro-Buddy study to assess the exposure of SARS-CoV-2 in youths. This substudy recruited participants between the summer of 2020 and the end of 2020 in the city district of Tübingen. Overall, 837 saliva samples were analyzed, including 504 from preschool children (aged 1 to 6) and 333 from primary school children (aged 6 to 10). In total, 12 participants tested positive for RBD-specific IgG in saliva, with a positivity rate of 1.6%. Interestingly, preschool children had a significantly lower prevalence of 0.4% ($p = 0.0047$, Fisher's exact test, two-tailed) compared to 3.0% in primary school children.

Similar age-dependent differences in SARS-CoV-2 specific IgG levels in serum and saliva have been described in other studies. Keuning et al. (2021) reported in a study in the Netherlands from April to October 2020, including 223 children attending medical facilities (93). They observed a reduced seroprevalence of 2.0% in younger children (0 to 10 years) compared to 4.2% in youths aged 10 to 17 (93). Similarly, a study by Stringhini et al. (2020) in Geneva found that only 0.8% of the children 5 to 9 years old ($n = 123$) had a positive ELISA result (serum, Euroimmun kit), while 9.6% of adolescents aged 10 to 19 years ($n = 332$) were positive (94). In the hospital-based study by Tönshoff et al. (2021), the infection prevalence in 2482 child-parent pairs (children 1 to 10 years old) was measured with serum-based immune assays (83). Participants were recruited in hospitals in the spring of 2020 in the southwest of Germany, in the same geographical region where the Coro-Buddy study was conducted (83). The difference in seroprevalence between the children age groups was minor, with a prevalence of 0.8% and 1.0% in children 1 to 5 years and 6 to 10 years old, respectively (83). Nevertheless, the overall estimated prevalence of the children (0.6%) was considerably lower than that of the parents (1.8%) (83). In summary, the estimated prevalence based on IgG levels in saliva and serum is generally lower in younger children compared to older children and adolescents. However, the mechanisms for these age-related differences remain unclear.

Several factors could have contributed to the age-dependent prevalence of SARS-CoV-2 at that time. Young children appear to have a more active and effective immune response, encountering more frequently new pathogens after losing maternal

immunity (95). Yang et al. (2021) reported for a study population aged 1 to 24 years that the total IgG and SARS-CoV-2 IgG levels, neutralizing activity, and avidity correlated negatively with age (80). Furthermore, other pathogens might induce cross-reactive immunity, potentially helping young children resist SARS-CoV-2. Viart et al. (2022) found a very low SARS-CoV-2 prevalence of 0.3% in France among 924 young children (median 4 years old), while 15.4% were convalescent for other respiratory viruses (predominantly rhinovirus) (96). Cross-reactive immunity or a more effective immune system could lead to more asymptomatic cases or make a SARS-CoV-2 infection in younger children less likely. Additionally, younger children might have smaller social groups, with less frequent social contacts, potentially limiting the spread of SARS-CoV-2. In the study of Glass et al. (2008), it was observed that in a school setting, older students were more assortative and spent more time with their contacts, potentially resulting in a higher transmission rate of diseases in teenagers (97).

Another influencing factor could be the gene expression of the ACE-2 receptor on epithelial cells of the respiratory system. In the publication by Bunyavanich et al. (2020), a significant correlation between age and ACE2 receptor expression levels was shown (98). The low expression levels found in the nasal epithelium of young children (age <10 years) (98) could potentially reduce virus binding and cell entry and lead to a reduced SARS-CoV-2 prevalence in young children.

While fewer studies focus on mucosal immunity by analyzing IgG and IgA in saliva, Dobaño et al. (2021) measured salivary RBD-specific IgG in 1907 children (aged 0 to 14 years) in the summer of 2020 in Spain, reporting a prevalence of 0.37% (99). Like in the Coro-Buddy study, Oracol saliva collection devices (Malvern, UK) were utilized. They were effective for the saliva collection of infants, as spitting can be difficult for them (99). The prevalence was overall lower than in the Coro-Buddy study, although a direct comparison of results between studies is difficult, as SARS-CoV-2 exposure and prevalence can vary considerably between locations and collection times. Interestingly, a later analysis by Dobaño et al. (2022) found that asymptomatic participants had higher Spike IgG levels than symptomatic individuals, indicating a potential protective effect of high IgG concentrations in the mucosa (100). Overall, no significant age-related differences in RBD-specific IgG levels were observed in the Coro-Buddy study.

During the Coro-Buddy study, the salivary RBD-specific IgG did not exceed the number of PCR-confirmed cases registered by the local health authorities. Compared to the PCR cases, an elevated number of IgG-positives was expected due to a higher rate of asymptomatic cases in youths (50,78). Young children (1 to 10 years old) registered in Tübingen accounted for 34 PCR cumulative cases, resulting in a prevalence of 0.5%. A modest difference in PCR-based prevalence was observed between preschool and primary school children of 0.4% and 0.7%, respectively. Overall, the PCR data followed a similar age-dependent trend, although not as strong as seen between the IgG prevalence of preschool and primary school children during the Coro-buddy study.

However, the gap between the PCR data from children 6 to 10 years old (0.7%) and the estimated IgG prevalence of 3.4% (95% CI 1.9 – 6.2) indicates an increased number of previously undetected, asymptomatic cases. Indeed, 11 out of 12 IgG-positive tested children were unaware of an infection and only 2 (17%) reported cold-like symptoms. These findings reinforce the hypothesis that many children had an asymptomatic SARS-CoV-2 infection. Still, about 40% of the participants self-reported cold-like symptoms 2-months before collection, predominately younger participants. According to the literature, infants and young children encounter new pathogens more frequently than older youths as they build up their immune defences (95). In conclusion, cold-like symptoms were an overall poor indicator for SARS-CoV-2 infections.

Potential exposure to SARS-CoV-2 by their respective households was self-reported by 3 out of 12 IgG-positive children. No clusters were apparent in the educational institutions. Data from the publication of Strich et al. (2021) and Tönshoff et al. (2021) indicate that children were more likely to be infected by household members than vice versa. However, among participants reporting a SARS-CoV-2 infection, only 1 out of 4 children was IgG-positive in saliva. Notably, 2 out of these 3 participants reported a SARS-CoV-2 positive test 5 to 6 months before collection, potentially resulting in waning antibody concentrations. These IgG levels could be too low to be detected in saliva, as the assay is less sensitive than plasma due to an overall lower IgG concentration. Notably, no PCR tests were reported, and first-generation antigen and antibody tests showed a wide span in sensitivity and specificity, potentially leading to an elevated risk of false positive results (101). Other explanations include rapid antibody degradation by mishandling of the saliva during sample collection or ELISA preparation.

Further limitations were the absence of T- and B-cell experiments, which are crucial for understanding the cellular immune response after SARS-CoV-2 infection. Without the collection of blood specimens, such experiments were not feasible. Furthermore, no sIgA measurements were performed at this point of the study (unpublished data, not shown). Compared to IgG, sIgA has a short lifespan, being detectable only 6 to 10 weeks after infection (102). For that reason, IgG measurements were favoured for detecting SARS-CoV-2 prevalence.

In summary, the ELISA for RBD-specific IgG in saliva was successfully implemented in the Coro-Buddy study, detecting the SARS-CoV-2 prevalence in youths. Interestingly, preschool children showed a significantly lower prevalence (0.4%) than primary school children (3.0%), following the evidence that younger children are less often seropositive for SARS-CoV-2 Wildtype. Most IgG-positive children were unaware of a previous SARS-CoV-2 infection, displaying no cold-like symptoms 6-months before the collection. Overall, cold-like symptoms have been a poor indicator of SARS-CoV-2 infection.

## 4.2. SARS-CoV-2 Antibodies Are Persisting in Saliva for More Than 15 Months After Infection and Become Strongly Boosted After Vaccination

Understanding antibody persistence and kinetics has been crucial for conducting longitudinal seroprevalence studies and assessing vaccine efficacy. While salivary antibodies received less attention compared to blood-based antibodies, they are suspected to contribute to protection against severe COVID-19 and reinfections (103,104). To shed more light on this topic, 72 adult participants who experienced a mild SARS-CoV-2 infection were followed up for up to 15 months. Results showed that most unvaccinated participants had persistent IgG levels in plasma (98%) and saliva (80%) that slowly diminished. Overall, IgG levels in saliva and plasma correlated greatly (Person’s correlation r = 0.73).

Durable and specific immune reactions have been described in other publications as well (103–105). Alkharaan et al. (2021) observed that plasma and saliva samples from patients with mild symptoms (n = 74) mostly retained SARS-CoV-2 specific IgG for up to 9 months after recovery (97% in blood and 88% in saliva) (104). The rate of anti-Spike IgG-positive participants remained high compared to NCP-specific IgG, which declined more rapidly in saliva (104). Similarly, in the publication of Chellamuthu et al. (2021), the salivary IgG levels remained stable in participants (n = 42) throughout the

study duration of 12 months (105). A strong correlation between IgG antibodies in serum and oral fluids was also detected (105). Interestingly, Hoschler et al. (2022) reported an almost identical factor between IgG antibodies in saliva and plasma of roughly 1 to 1000, with IgG levels being lower in saliva (106). In summary, there is strong evidence that even a mild SARS-CoV-2 infection can induce long-lasting IgG antibodies that persist for over a year in saliva and blood. A strong correlation between IgG in plasma and oral fluids makes saliva a valid source for determining the blood IgG status.

However, it is difficult to predict a threshold for antibody-induced protection against SARS-CoV-2, especially with the appearance of new VOC strains. Additional factors, such as antibody avidity and cellular immune response, which can impact the immune response, were not incorporated into the analysis. Nevertheless, in a recent study by Kenny et al. (2023), a threshold of protection was estimated with an RBD-specific IgG level of 456 BAU/ml in convalescent and unvaccinated individuals. A significant host viral neutralization against the WT SARS-CoV-2 strain was predicted with 80% accuracy (107). A cut-off of 456 BAU/ml would translate to 129 µg/ml RBD-specific IgG, according to an applied international WHO control 20/136 (1000 BAU/ml equals 283 µg/ml in the in-house ELISA). Most convalescent participants were below that threshold, indicating that an antibody boost by vaccination after the initial infection would be beneficial. For saliva, similar thresholds have not been reported in the literature.

During the follow-up of the study, about 58% (42/72) of the participants got vaccinated against SARS-CoV-2. The majority received their first dose from BioNTech (n = 22), AstraZeneca (n = 13) or Moderna (n = 7). The comparison between the time points before and 1-month after vaccination illustrates a rapid increase in IgG in both plasma (72-fold) and saliva (46-fold). In the publication of Doria-Rose et al. (2021), antibody persistence has been reported for 6-months after the second vaccination of Moderna (108). Similarly to our findings, IgG levels were high after immunization, and the regression lines were flat (108). Interestingly, participants who were IgG negative before reached lower IgG levels post-vaccination, indicating a reduced immune response or a potentially false-positive SARS-CoV-2 rapid test. However, this trend was not significant, and participant numbers were low. Due to relatively low participant numbers for each vaccination type, a comparison between IgG levels was not done.

For the functional analysis of SARS-CoV-2 specific antibody levels, a surrogate neutralization assay (NeutraLISA) was performed. Interestingly, after 4 months post symptom onset, only 34% (17/50) of the convalescent participants had neutralizing antibodies in plasma. However, participants receiving vaccination had highly elevated neutralization activity of 100% (33/33), with a mean inhibition of 96% vs 10% in the unvaccinated group. High IgG levels in plasma were found to correlate with neutralizing activity of the surrogate test (Pearson correlation, r = 0.70, see Figure 3B). The low neutralizing activity of antibodies in convalescent individuals also indicates the potential benefit of an immunization after the first infection of SARS-CoV-2.

The advantages of the surrogate neutralization tests over the conventional plaque reduction neutralization tests (PRNT) are that no BSL-3 lab is required, and the test takes only a few hours instead of days (109). In the publication of Graninger et al. (2023), multiple surrogate assays were tested and compared with results from the live-virus neutralization test. They found substantial differences in sensitivity and specificity between assays, with the applied NeutraLISA test being one of the best-performing assays in the ROC curve analysis (110). Differently, in the publication of Hoffmann et al. (2022), the sensitivity of the NeutraLISA assay was comparably low (50.0 – 67.9%) while maintaining a high specificity (100%) compared to the PRNT results. A limitation of this method was that it was not sensitive enough to detect neutralizing antibodies in saliva due to the overall lower IgG levels.

In summary, mild SARS-CoV-2 infections induced long-lasting IgG antibodies that persisted over one year in plasma and saliva with correlating IgG levels. However, antibody-induced protection is not possible to estimate, especially with newly emerging SARS-CoV-2 variants. With decreasing IgG antibody levels, immunization can be beneficial to boost the immune system. Overall, convalescent participants who received vaccination displayed highly elevated IgG levels in plasma and saliva. Additionally, an enhanced antibody performance in plasma was observed in the surrogate neutralizing test.

## 4.3. Saliva and Plasma Antibody Levels in Children and Adolescents After Primary Infection With Omicron Variants of SARS-CoV-2 Infection in Germany

With the emergence of the Omicron variant, the course of the SARS-CoV-2 pandemic fundamentally changed at the end of 2021. Unlike previous strains, Omicron displayed

the ability to efficiently infect vaccinated and convalescent individuals, leading to a massive outbreak in Germany between January and May 2022. Many young children had not received vaccination yet and were exposed for the first time to the SARS-CoV-2 virus, specifically the Omicron variant. Limited information was available regarding this primary Omicron immune response. Astoundingly, children with a positive PCR test during the Omicron outbreak (n = 173) had significantly lower IgG levels in saliva compared to participants previously infected with an earlier variant (pre-Omicron) ($p < 0.001$ by Wilcoxon rank sum test). Specifically, 82% of primary Omicron convalescents displayed RBD IgG levels below the positivity threshold. This phenomenon was also observed with IgG reactive to other Omicron antigens like the trimeric Spike (Tri) and NCP protein. Additional experiments on IgG in plasma displayed a similar reduction in IgG levels for primary Omicron-infected children. A hypothesis would be that primary Omicron infections induce a weak humoral response in youths, resulting in a diminished antibody production. This phenomenon might be connected to the immune evasive behaviour of the Omicron variant.

Extensive mutations in the Omicron spike and non-spike proteins have been described to contribute to immune evasive properties (25,111,112). Previously neutralizing antibodies cannot interact well with the RBD and N-terminal domain of the spike protein due to mutations at the binding sites (111,113). Therefore, the potency of preexisting antibodies was tremendously reduced (111,113). Additional mutations on the N protein, M protein, and non-structural proteins (nsp) have been reported to suppress the host immune response, potentially increasing virus survival (25). Furthermore, accessory proteins (ORF proteins) were reported to inhibit interleukins and interferons, altering signalling pathways regulating the immune system (112). In the publication of Hossain et al. (2022), non-spike protein mutations induced host immune evasion by disrupting critical protein synthesis, preventing autophagosome and lysosome fusion and leading to the deactivation of natural killer cells (112). Interestingly, natural killer cells do not only destroy virus-infected cells but are a crucial connector between the innate and adaptive immune response (114). Natural killer cells have been described to be involved in dendritic cell maturation and activation, indirectly influencing TH1 and B-cell responses (114). Overall, Omicron-mediated host immune suppression targets predominately parts of the innate immune response, which could have downstream effects on adaptive immunity and antibody production.

In general, there has been limited information on IgG levels after primary Omicron infections, as most adults were previously vaccinated or had a SARS-CoV-2 infection in the past. Excluding this publication, no reports were found on the salivary IgG response in children after a primary Omicron infection. Nevertheless, similar findings to those of the Coro-Buddy study have been described for plasma by Dowell et al. (2023). They found low levels of humoral response one month after primary Omicron infection in youths (115). After a primary Omicron infection (n = 20), antibody levels in plasma were low and displayed weak neutralization capabilities against the Omicron variant, with a quarter having no detectable neutralizing activity (115). Interestingly, the cellular immune response by T-cells was much more robust and equal to pre-Omicron convalescent participants (115). Similarly, a following Omicron infection or a previous contact with SARS-CoV-2 elicited higher antibody levels (115).

In the publication of Toh et al. (2022), significant differences in antibody levels between pre-Omicron and Omicron variants in unvaccinated youths were also described (116). Omicron infection resulted in an average S1-IgG antibody level in plasma of 46 BAU/ml (16% inhibition in a surrogate neutralization assay) vs 435 BAU/ml (77% inhibition) for the Delta variant ($p<0.001$, Wilcoxon rank sum test). However, most of the Delta (35/35) and Omicron-infected children (13/16) displayed an IgG seroconversion after 36 days.

A study conducted in Taiwan by Huang et al. (2023) assessed the humoral immune response in plasma of children after an Omicron infection (117). Contrary to the Coro-Buddy findings, relatively high antibody levels were found in primary Omicron convalescent children (n = 67), especially in the age group 6 months to 2 years old (117). However, 76% of the recruited children were admitted to the hospital, and 16.5% were even in the intensive care unit (117). Thus, the symptoms were much more severe than in the Coro-Buddy study, and the participants displayed an increased proportion of other diseases and comorbidities (117). These findings might indicate that severe Omicron infections can induce a robust humoral response against the Omicron variant in youths. However, a limitation of the study was that antibody levels from pre-Omicron convalescent participants were not measured and directly compared to primary Omicron samples, making a direct comparison to the Coro-Buddy results difficult.

An important factor when comparing the IgG levels between the pre-Omicron and Omicron groups is the timespan between the SARS-CoV-2 test and the saliva

collection. IgG antibodies naturally wane over time, resulting in lower IgG levels after an extended period. Interestingly, the number of days between the COVID test and sample collection was higher in the pre-Omicron group (median 175 days) compared to the Omicron cohort (median 54 days (RBD WT) or 84 days (RBD BA.1)). This, in turn, should lead to comparably lower IgG levels in the pre-Omicron convalescent group, even though the opposite has been observed. The anti-SARS-CoV-2 IgG response in saliva and serum peaks after 16 – 30 days PSO and can remain for more than 100 days in both specimens (103). Therefore, a complete decay of RBD-specific IgG antibodies in the primary Omicron convalescent cohort is unlikely.

However, due to low levels of Omicron-specific antibodies, the risk for reinfections might increase, leaving Omicron convalescent individuals vulnerable to new Omicron variants. An increased reinfection risk through Omicron is further supported by a publication by Medits et al. (2022), finding profoundly reduced cross-neutralization profiles in primary BA.1 and BA.2 Omicron-infected adults (118). These findings indicate a narrow neutralizing specificity of antibodies that might not be effective against other Omicron variants (118).

Indeed, during the secondary sample collection of the Coro-Buddy study in 2022 (n = 20), some participants (n = 3) reported a secondary Omicron infection four to five months after the first infection. In the study of Carazo et al. (2023), breakthrough infections were analyzed in over 37.000 adults in Canada who tested positive for a BA.2 Omicron (119). Interestingly, 6.7% had a previous pre-Omicron and 1.7% a BA.1 Omicron infection, indicating a higher rate of protection from reinfection in BA.1 convalescent individuals (119). In comparison, Malato et al. (2022) described a lower rate of protection efficacy of 75% (120). The risk of infection with the BA.5 variant in a BA.1/BA.2 convalescent highly vaccinated adult cohort was investigated (120). Interestingly, in Omicron convalescent youths (5 – 11 years), a similar rate of 80% protection after 3 months and 54% after 6 months was found by Lin et al. (2023) (121). Younger children (0 – 4 years) displayed similar protection against Omicron reinfection of 77% 3 months post Omicron infection and 65% after 6 months (121). For reference, youths had a protection rate after primary vaccination of 60% after the first month and 34% after 4 months (121). In summary, while Omicron convalescent individuals appear to have some protection against reinfection, the risk remains relatively high compared to pre-Omicron variants. Moreover, the protection efficacy decreases notably after a few months.

A potential limitation of the study was that most of the participants with a primary Omicron infection were younger (mean 6 years old, range 1 to 13 years) than the pre-Omicron convalescent individuals (14 years, range 11 to 16). During the Omicron outbreak in early 2022, most older youths had already received vaccination, whereas no official recommendation had been issued for younger children. Potentially, this age difference could influence the immune response. However, in a similar study by Toh et al. (2022), the lower antibody response of Omicron-infected children was not associated with age (116).

Additionally, the SARS-CoV-2 variant causing the infection was not determined by PCR. Instead, estimations were based on the predominant variant found by the RKI (weekly reports) at the time of infection. This approach carries a minor chance that other variants were falsely included. Another limitation of the study applies to the prevalence detection. Even though a high prevalence of 20% was detected in the Coro-Buddy study during the Omicron outbreak in early 2022 in Germany, many individuals with a positive PCR test did not express SARS-CoV-2 specific-IgG in saliva and severely reduced levels in plasma. This phenomenon led to an under-representation of convalescent individuals.

In summary, a significant difference in antibody expression between pre-Omicron and Omicron primary convalescent children was observed in saliva and plasma. There is evidence that the Omicron strain has immune evasive properties that result in a reduced humoral response, potentially leading to an increased risk of reinfection by new SARS-CoV-2 variants.

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Appendix (publications)

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# Non-Invasive Antibody Assessment in Saliva to Determine SARS-CoV-2 Exposure in Young Children

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Saliva is a body fluid with hitherto unused potential for the assessment of SARS-CoV-2 antibodies. Specific antibodies can indicate a past SARS-CoV-2 infection and allow to estimate the proportion of individuals with a potential protective immunity. First, we carefully characterized plasma samples obtained from adult control groups with and without prior SARS-CoV-2 infection using certified reference ELISAs. Simultaneously collected saliva samples of confirmed convalescent and negative individuals where then used to validate the herein newly developed ELISA for the detection of SARS-CoV-2 IgG antibodies in saliva. The saliva ELISA was applied to assess SARS-CoV-2 exposure in young children (N = 837) in the age between 1 and 10 years in Tübingen, Germany, towards the end of the first pandemic year 2020. Sensitivity and specificity of the new saliva ELISA was 87% and 100%, respectively. With 12% of all Tübingen children sampled *via* their respective educational institutions, estimates of SARS-CoV-2 antibody prevalence was 1.6%. Interestingly, only 0.4% preschool kids were positive compared to 3.0% of primary school children. Less than 20% of positive children self-reported symptoms within two months prior to saliva sampling that could be associated - but not exclusively - with a SARS-CoV-2 infection. The saliva ELISA is a valid and suitable protocol to enable population-based surveys for SARS-CoV-2 antibodies. Using non-invasive sampling and saliva ELISA testing, we found that prevalence of SARS-CoV-2 antibodies was significantly lower in young children than in primary school children.

**Keywords: SARS-CoV-2, saliva, antibodies, children, epidemiology, prevalence**

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## INTRODUCTION

The COVID-19 pandemic has been keeping the world on its toes for more than one year. Many countries have - and continue - to experience repeated periods of lockdown, and restrictions of their social and economic life. Restrictions and closures of day-care facilities and schools can adversely impact a child's educational opportunities, supports and overall well-being. From early on, the children´s role in the COVID-19 pandemic was a matter of intensive debate, as there was a lack of

relevant data on this novel disease. Fortunately, evidence accumulated relatively rapidly that children and adolescents without underlying chronic disease are rarely suffering from severe clinical manifestations and case fatality is very low (1). However, the contribution of minors to SARS-CoV-2 transmission is difficult to assess and depends on many parameters that vary over time and situational contexts. In 2020, the first pandemic year, children were reported to be less likely to become infected with SARS-CoV-2 compared to adults and elderly. For infants, the reported number of cases were even lower (2–4).

Public records on SARS-CoV-2 cases do not fully inform on the infection rate in children, as PCR testing is largely performed on symptomatic individuals, who are mostly adults. In contrast, children who contract SARS-CoV-2 are commonly mild diseased or even asymptomatic (5–7). Antigen tests have become more widely used since the start of 2021 and might unveil more infections in paediatric populations. Seroprevalence studies detecting antibody responses to SARS-CoV-2 as a marker of a preceding infection, allows for the estimation of the proportion to which the population has been infected (and/or vaccinated). This aids in monitoring of the pandemic´s progression and provides an approximate quantity of individuals with potential protective immunities. Although antibody concentrations are typically assessed in blood, it is known that participants are often reluctant to phlebotomy if not medically required – particularly young children and their parents. An alternative approach is to measure the SARS-CoV-2 specific antibody response in saliva. Sampling of salivary specimens is non-invasive, feasible in non-medical settings, is well tolerated by children and their parents and allows for mass screening. Studies to detect antibodies in saliva have been successfully used to monitor exposure to Norovirus (8), HIV (9) and also malaria parasite infections (10), as these antibody profiles match well with those found in blood. The first SARS-CoV-2 studies conducted confirm this also for previous SARS-CoV-2 infections as well as COVID-19 mRNA vaccination (11–13). Although concentrations in blood are considerably higher (14), salivary antibodies are in fact reactive to the SARS-CoV-2 spike protein (S protein) and to the receptor-binding domain (RBD). Interestingly, both S protein and RBD-specific IgG profiles are more consistent and higher in titres compared to secretory IgA in salivary samples and SARS-CoV-2 IgGs are maintained at least for three months (15). No standardized protocol or certified commercial enzyme-linked immunosorbent assay (ELISA) for the detection of SARS-CoV-2 antibodies in saliva is currently available (by May 2021).

We describe here the development of an in-house ELISA for the detection of SARS-CoV-2 RBD reactive IgG antibodies in saliva, the 'saliva ELISA'. Assay performance was validated using paired plasma and saliva samples obtained from adult control groups, with their plasma samples carefully characterized using certified reference ELISAs. The saliva ELISA was then applied to non-invasively collected saliva samples obtained in autumn 2020 from young children in the age between 1 and 10 years participating in an ongoing prospective, longitudinal study to determine the incidence of SARS-CoV-2 infections in children and adolescents in the University city of Tübingen, Southwest Germany.

# MATERIALS AND METHODS

## Study Design and Study Population

To establish and to validate the new saliva ELISA protocol, plasma and saliva was simultaneously collected from control groups that were adults with a prior SARS-CoV-2 infection (convalescent group) or without prior SARS-CoV-2 infection but collected under the pandemic situation in 2020 (negative group). Previous SARS-CoV-2 infection was confirmed by PCR or ELISA documentation. Banked plasma collected from adults in the years before 2020 served as an additional unexposed, negative control (prepandemic group).

Prevalence of SARS-CoV-2 antibodies was assessed in saliva collected in autumn 2020 in frame of the Coro-Buddy study from children aged 1 to 6 years (preschool cohort) and from children 6 to 10 years old (primary school cohort). Additionally, saliva from (mainly) accompanying parents and tutors was collected (adult cohort). Participants or participant´ parents and their household members were surveyed using a questionnaire for positive SARS-CoV-2 tests 6 months prior to sampling and for COVID-19 associated symptoms (fever > 37.5°C, sore throat, cough, rhinitis, others) occurring 2 months prior to sampling. From the few individuals of the adult cohort tested positive for salivary SARS-CoV-2 IgG, blood and an additional saliva sample were collected to reconfirm SARS-CoV-2 IgG positivity by EUROIMMUN ELISA (plasma) and by saliva ELISA. Sample size for the children cohort was based on the assumption of an overall incidence of 509/100.000 in the larger region (Landkreis Tübingen) in April 2020. Randomization was not applicable.

Coro-Buddy is a currently ongoing (by May 2021) observational, prospective study with the overall aim to longitudinally assess prevalence of SARS-CoV-2 antibodies in saliva as a proxy for seroprevalence at three timepoints within 12 months in 1,850 children aged 1 to 18 years in Tübingen, Germany. Here, only the results for the young children (1 to 10 years old) and from adults are reported. Coro-Buddy study protocol includes an additional control group of adults who were previously infected with SARS-CoV-2.

## Sample Collection and Processing

Blood (9 ml) was collected using lithium heparin monovettes and obtained plasma was stored at -20°C. Saliva was collected by Oracol S14 saliva collection device (Malvern Medical Developments, UK) by gently brushing the gum line for 2 min or by drooling into a simple plastic tube (multi-purpose containers 30 ml Greiner Bio-One ref. 201150). Saliva samples were kept on ice for a maximum of 3 hours before being further processed. Oracol S14 saliva collection device and plastic tubes were centrifuged at 2500 rpm for 6 to 10 min, respectively, to remove any debris. Supernatant was transferred into a 2 ml microtube and inactivated solvent/detergent treatment by using tri-n-butyl phosphate (TnBP) and Triton X-100 at final

concentrations of 0.3% and 1%, respectively. Samples were stored at -20°C.

## Antigen Expression

The pCAGGS plasmid encoding for the SARS-CoV-2 receptor binding domain (RBD) protein was kindly provided by Florian Krammer (Icahn School of Medicine at Mount Sinai, New York) (16). The RBD sequence encodes for amino acids R319 to F541 of the spike protein plus a C-terminal His tag. Recombinant RBD protein has a molecular weight of 27.5 kDa (without glycosylation). Human embryonic kidney Expi293F™ Cells (HEK cells, Thermo Fisher; ref. A14528) cells were transfected with pCAGGS plasmid using ExpiFectamine™ 293 Transfection Kit (Gibco™; ref. A14525). Transfection and supernatant harvest were performed according to the manufacturer's manual. Supernatant was purified by an ÄKTA chromatography system using a HisTrap HP 5 ml column (GE Healthcare, ref. 17524802). Protein size and quality control of the recombinant RBD protein were performed by SDS-Page and Western blot analysis. RBD protein concentration was diluted to 2 mg/ml in 1x PBS supplemented with protease inhibitors (Roche; ref. 11697498001).

## ELISA for Saliva Samples

To measure SARS-CoV-2 IgG antibody concentrations in saliva, a new ELISA was established. Prior to the final assay conditions the following blocking reagents were tested: The Blocking solution (Candor; ref. 11001L), Smart Block (Candor; ref. 113125), Plate Block (Candor; ref. 112125), 5% non-fat dried milk in 1x PBS (Roth; ref. T145.2), 1x ROTI Block buffer (0.5x in ddH2O, by Roth; ref. A151.2), Thermo Scientific Blocker Casein in PBS (Thermo Scientific; ref. 37528), Thermo Scientific Pierce Protein-Free (PBS) Blocking Buffer (Thermo Scientific; ref. 37572), gelatin from cold water fish skin (Sigma-Aldrich; ref. G7041-100G), 3% BSA in 1x PBS (SERVA; ref. 11930.04). The final assay conditions were as follows: SARS-CoV-2 RBD antigen was diluted in 1x PBS to a final concentration of 2 µg/ml and 50 µl was added per well to coat Costar 96 well microtiter high binding plates (ref. 3590, Corning). After overnight incubation at 4°C, the wells were washed once with 1x PBS and blocked with 200 µl of The Blocking Solution (Condor Bioscience GmbH) for 2 hours at room temperature on a microplate shaker (700 rpm). Subsequent washing steps were repeated 3x with PBS/0.1% Tween20. Saliva and control samples were diluted 1:3 to 1:27 using The Blocking Solution and 100 µl was added per well and incubated for 1 hour. IgG antibody capture was detected by 1:20,000 diluted biotinylated anti-human IgG (ref. 109-065-008, Jackson Immuno Research Laboratories) and 1:1000 Avidin-HRP (Biolegend ref. 405103). Both reagents were dissolved in 1x ROTI Block buffer (Roth) and incubated for 1 hour and 30 min, respectively. For visualization, 100 µl TMB substrate solution was added, and the reaction was stopped using 50 µl 1M HCl. The plate was read at 450 nm and 620 nm with a microplate reader (CLARIOstar, BMG LABTECH). Concentrations (ng/ml) of salivary SARS-CoV-2 IgG were estimated by comparison against highly pure human IgG (standard) which was precoated as dilution series on separate wells on the same plates (ref. 31154, ThermoFisher). This approach has previously been reported by others (17, 18).

## ELISA for Plasma Samples

Plasma samples were analysed using the EUROIMMUN SARS-CoV-2 IgG ELISAs kit detecting antibodies binding to SARS-CoV-2 Spike protein domain S1 in accordance with the manufacturer's instructions. Plasma was diluted in a provided sample buffer, added to antigen-coated microtiter wells, and then incubated at 37°C for 1 hour. Plates were washed and then a conjugated solution was added and incubated at 37°C for 30 min. After a second wash step, the substrate solution was added and incubated at RT for 30 min. Finally, 0.5 M sulfuric acid stop solution was added and absorbance of sample wells measured immediately at 450 nm and 630 nm using the CLARIOstar microplate reader (BMG), with output reports generated with optical density (O.D.) at 630 nm subtracted from O.D. at 450 nm. Data were then analysed as recommended by the manufacturer and results reported as a ratio. Following EUROIMMUN specifications the cut-off for SARS-CoV-2 IgG positivity is set at ≥ 1.1 ratio, intermediate antibody concentration is at 0.8 to 1.1 ratio, and negative is defined as < 0.8 ratio. To confirm findings from EUROIMMUN assays, the COVID-SeroIndex, Kantaro SARS-COV-2 IgG Antibody ELISA was performed as second ELISA.

## Data Analysis

Graphics were generated with Graph Pad Prism (Version 9.1) and RStudio (Version 1.2.5001), running R (version 4.0.4.). Proportion of correctly classified samples was calculated for the selection of the cut-off value for the IgG saliva ELISA and ROC-curve analysis was used to describe assay performance and determine the sensitivity and specificity of the test. A $Chi^2$-test was performed to estimate the capacity of the saliva ELISA to discriminate the positive or negative results from the serum ELISAs. The two-way ANOVA trend-test was used to determine if an existing trend for the stability of the saliva specimen at RT, 4°C and multiple freeze-thaw cycles exists. Pairwise Pearson's correlation coefficient was used to calculate the correlation of saliva samples at RT and 4°C.

Simple descriptive statistics (total number and proportion) were used to describe symptoms, reported previous infections, and prevalence of SARS-CoV-2 antibodies. The estimated prevalence of SARS-CoV-2 antibodies in saliva and its 95% confidence interval were computed and adjusted using the R package epiR Version 0.9-43, which uses the Rogan Gladen estimate for estimating the prevalence and the code provided by Reiczigel et al. (19) for computing the confidence intervals. The adjusted prevalence was calculated considering an imperfect test with parameters of specificity 100% and sensitivity 87%. The prevalence ratio was estimated using the relative risk function implemented in Prism 9.1, and the CI values were estimated using the Koopman asymptotic score.

# RESULTS

## Saliva ELISA Validation

We developed an in-house ELISA to detect IgG antibodies reactive to SARS-CoV-2 RBD domain in saliva, the saliva ELISA. RBD antigen was expressed in HEK cells and a

thorough analysis of 9 different blocking reagents was done (**Supplementary Figure 1**). The final protocol is specified in the Methods section.

To characterize the performance of the saliva ELISA to identify SARS-CoV-2 specific antibodies in saliva, a set of adult control groups was selected consisting of paired plasma and saliva samples obtained from i) COVID-19 convalescent individuals (convalescent group) confirmed by either PCR or ELISA documentation (n = 75, median age = 28 years, range of 19-75 years, sex= 51 women/24 men), ii) individuals without known history of SARS-CoV-2 infection (negative group, n = 108, median age = 30 years, range of 13-61 years, sex = 71 women/37 men), and iii) a set of prepandemic plasma samples (but no saliva) collected in the years 2015-2019 (prepandemic group, n = 32, median age = 32 years, range of 23-53 years, sex = 10 women/22 men). None of the COVID-19 convalescent individuals were hospitalized during the disease period. Samples of the convalescent group were collected at 6.5 months (median: 196 days, 95% CI: 185 – 203 days) after symptom onset.

Prior to their application for validating the saliva ELISA, plasma samples of the control groups were all confirmed for positivity or negativity by SARS-CoV-2 serology. Therefore, all plasma samples of control groups were assessed by a certified commercial EUROIMMUN ELISA to quantify IgG reactive to the S1-domain of the of SARS-CoV-2 spike protein. Of the convalescent group 48/75 (64%) individuals were plasma IgG positive at the time of sampling (6.5 months after symptom onset). Intriguingly, in the negative group, 1/108 individuals had a positive SARS-CoV-2 IgG titre which was probably due to an asymptomatic, undetected infection (**Figure 1**). No cross-reactivity (0/32) to the S1-domain was found in prepandemic, banked plasma samples. A second ELISA test, COVID SeroIndex detecting RBD domain reactive IgGs, was done to confirm EUROIMMUN results only. Here, 47/48 plasma samples from the convalescent group were IgG positive and 99/104 negative group were confirmed to be seronegative.

Individuals with double-confirmed (EUROIMMUN plus COVID/SeroIndex) positive (convalescent group) or negative (negative group) SARS-CoV-2 serology were selected (**Supplementary Figure 2**), and their saliva samples served to validate the saliva ELISA (see below). Individuals with discordant EUROIMMUN/COVID SeroIndex outcomes were excluded from saliva ELISA performance assessment. For completeness, **Supplementary Tables 1**, **2** display the discordant reference ELISA results and their respective saliva ELISA outcomes.

From these double-confirmed control groups simultaneously sampled respective saliva (convalescent group n = 47, negative group n = 99) were used to define the performance of the saliva ELISA. The cut-off for salivary SARS-CoV-2 IgG positivity was determined by the combination of the proportion of correctly classified samples and the highest specificity, which was reached at 6.3 ng/ml. With this cut-off, all plasma confirmed negatives were also negative in the saliva ELISA (99/99). Of the 47 plasma confirmed IgG positives 41 (87%) individuals were found positive for salivary SARS-CoV-2 IgG (**Table 1**). Specificity and sensitivity of the saliva ELISA were 100% (95%CI: 96%-100%) and 87% (95%CI: 75%-94%), respectively (**Figure 2**). The proportion of correctly classified samples was 0.95 (95%CI: 0.91 – 0.98). The area under the ROC-curve was 0.995 (95%CI: 0.988 – 1.0) indicating that the convalescent individuals could be correctly differentiated from the negative individuals with high confidence ($p<0.0001$). Positive and negative predictive values were above 98% irrespective of the assumed COVID-19 prevalence (**Table 2**).

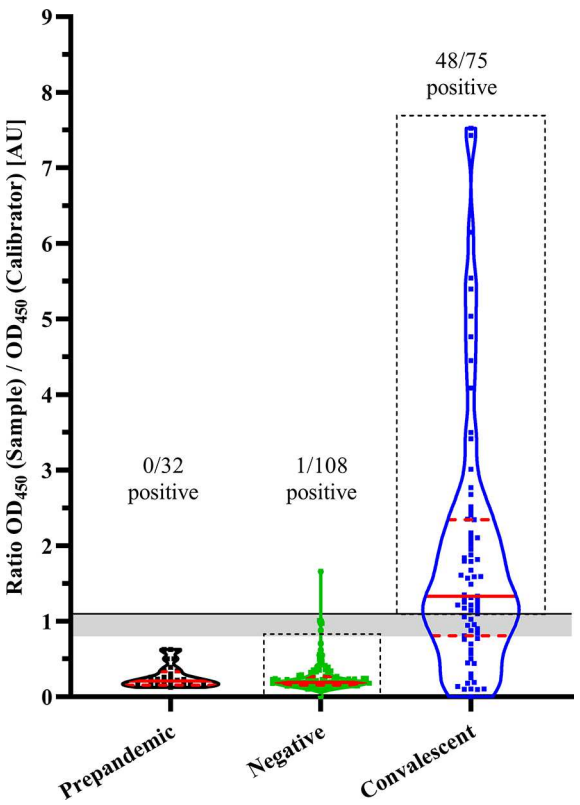


**FIGURE 1** | SARS-CoV-2 IgG levels in plasma of adult control groups. IgG concentrations reactive to SARS-CoV-2 were estimated by commercial ELISA (EUROIMMUN). Results are shown as violin plots. Prepandemic: plasma collected 2015-2019, Negative: Individuals without (known) SARS-CoV-2 infection, Convalescent: Individuals with previous COVID-19. Solid line: Cut-off for SARS-CoV-2 IgG positivity; grey area: intermediate antibody concentration; solid red lines: median; dashed red lines: quartiles. Black dashed-line boxes highlight selected individuals for subsequent ELISAs.

To determine reproducibility of the saliva ELISA, intra- and inter-assay precision parameters were estimated. For these

**TABLE 1** | Contingency table for saliva ELISA indicating the number of participants used to define the performance of the assay.

| Control groups/Saliva ELISA | Convalescent, n (%) | Negative, n (%) |
|---|---|---|
| Total, n | 47 (100) | 99 (100) |
| Positive, n | 41 (87) | 0 (0) |
| Negative, n | 6 (13) | 99 (100) |

*Chi²-test (two-tailed): $p < 0.0001$.*

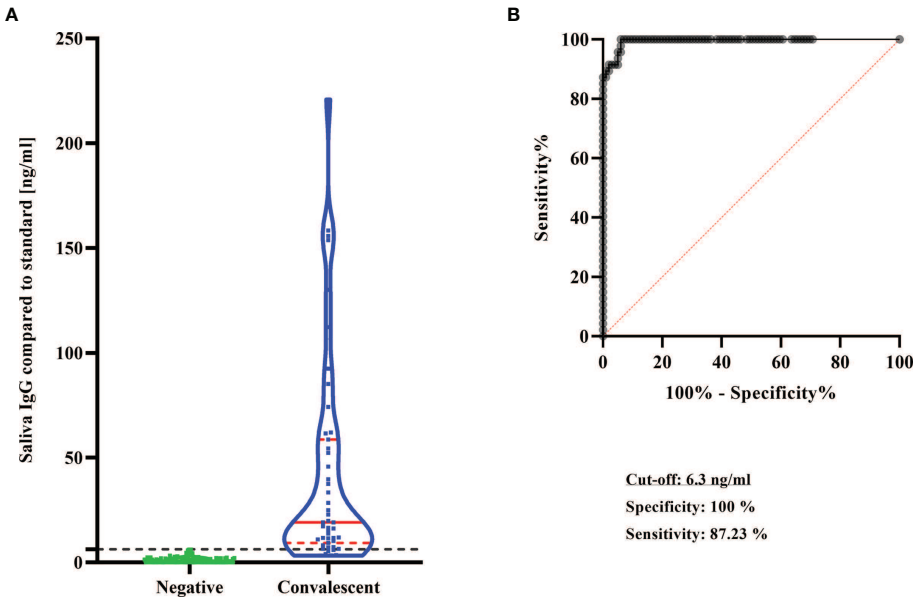


**FIGURE 2** | Performance of the saliva ELISA. **(A)** SARS-CoV-2 IgG concentrations in saliva in the negative group (n = 99) and in the convalescent group (n = 47). Results are shown as violin plots. Solid red lines: median; dashed red lines: quartiles, Dashed black line: cut-off of SARS-CoV-2 IgG positivity. **(B)** ROC curve analysis to determine diagnostic test performance of saliva ELISA.

assays, samples from the convalescent group with salivary IgG covering titres from high (80 ng/ml) to below the positivity threshold of 6.3 ng/ml were chosen. The coefficient of variation (CV) of the inter-assay ranged from 3% -13% which is well below the acceptable 15% for diagnostic assays (20) (**Figure 3**). The CV for the intra-assay repeatability assessed with dilutions of 1 to 3 to 1 to 6561 from a convalescent individual´s sample remained below 10%.

Saliva is a complex and non-sterile body fluid. Stability of IgG antibodies during storage and after repeated freeze-thaw cycles was assessed. Saliva samples were stored for up to 5 days either at room temperature (RT) or at 4°C. SARS-CoV-2 IgG remained largely stable when saliva was stored at 4°C ($p = 0.1$, one-way ANOVA), whereas storage at RT was detrimental to salivary IgG, RBD-specific IgG degraded over time ($p<0.0001$, one-way ANOVA, test for trend; **Figure 4A**). Therefore, all saliva specimens were stored at 4°C immediately after sampling and frozen after deactivation by solvent/detergent treatment within 6 hours. Freezing and thawing of saliva was not detrimental to salivary SARS-CoV-2 IgG concentrations that remained constant through 5 freeze-thaw cycles (**Figure 4B**).

**TABLE 2** | Saliva ELISA performance characteristics.

| Assay parameter | | | |
|---|---|---|---|
| Sensitivity in % (95%CI) | 87.2 | (0.75 to 0.94) | |
| Specificity in % (95%CI) | 100 | (0.96 to 1.00) | |
| Predictive values: | | | |
| *Disease prevalence*:* | *0.5%* | *5%* | *10%* |
| PPV in % | 100 | 100 | 100 |
| NPV in % | 99.9 | 99.3 | 98.6 |
| Accuracy in % | 99.9 | 99.4 | 98.7 |

**Positive and negative predictive values (PPV, NPV) were calculated for assumed COVID-19 prevalence.*

## Salivary SARS-CoV-2 Antibodies in Children

The Coro-Buddy study is a prospective, longitudinal, observational study on prevalence of salivary SARS-CoV-2 antibodies in children aged 1 to 18 years living in Tübingen, Germany. A total of 1,850 children were enrolled *via* their respective educational institution located in Tübingen, and saliva sampling on site is ongoing *via* the study team. The study began in July 2020 with 3 sampling periods planned - after the first pandemic wave 2020, before winter 2020/2021 and a last sampling period in spring 2021. Here, we report interim data for the prevalence of SARS-CoV-2 antibodies in saliva sampled between September 9$^{th}$ and December 2$^{nd}$, 2020 from 837 children aged between 1 and 10 years old, representing 12% of all children in this age group in Tübingen (**Table 3**, **Supplementary Table 3**). Cumulative SARS-CoV-2 cases (PCR) until early December 2020 in Tübingen were 1.4% (1,249 cases) and 0.5% (34 cases) for the young children only (**Table 4**).

Infants (1 to 6 years) were enrolled in 27 randomly selected preschool facilities and saliva was collected from 504 kids, whereas 333 children (6 to 10 years) were sampled in 6 primary schools. Accompanying parents and tutors were also invited to participate and 308 adults from preschool facilities and 75 from primary schools provided a saliva sample. Roughly 40% in all the cohorts (children and adults) self-reported cold-like symptoms (fever > 37.5°C, sore throat, cough, rhinitis, others)

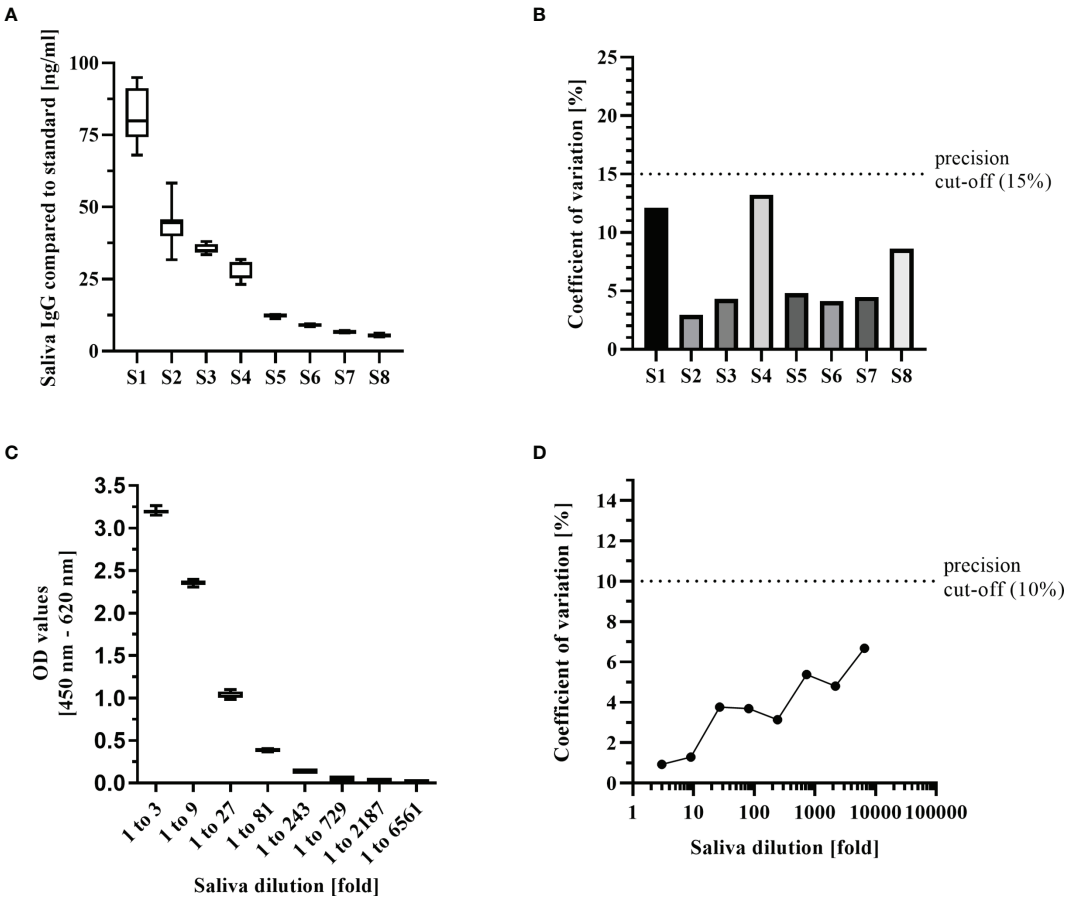


**FIGURE 3** | Key performance indicators of the saliva ELISA. **(A)** Inter-assay precision was estimated by measuring 8 samples in triplicates on 3 different days. Samples were selected to represent a range of IgG concentrations from high to negative. Results are shown as boxplots (median with interquartile range, whiskers represent 1.5x interquartile range). **(B)** The coefficient of variation of the inter-assay was calculated for each of the 8 samples. **(C)** Intra-assay precision was estimated by measuring a positive sample in 12 replicates with 8 3-fold dilutions and represented as boxplots. **(D)** Coefficient of variation of the intra-assay was calculated for each of the 8 dilutions.

within the 2 months prior to sampling (**Table 3**). A positive SARS-CoV-2 test within 6 months before sampling was self-reported by 2/504 (0.4%) preschool children and by 2/333 (0.6%) of the primary school children. Reported infection rates in adults were slightly higher at 6/383 (1.6%).

Saliva samples were analysed by the established and validated saliva ELISA used to determine positivity rates and titres of IgG specific to the RBD protein of SARS-CoV-2. Estimated prevalence of salivary SARS-CoV-2 IgGs adjusted for the sensitivity and specificity of the saliva ELISA was 1.6% (95%CI: 0.9%-2.9%) for all children, 0.5% (95%CI: 0.1%-1.7%) for preschool kids, and 3.4% (95%CI: 1.9%-6.2%) for primary school children (**Table 4**). Amongst the preschool cohort 2/504 (0.4%) saliva samples were positive for SARS-CoV-2 reactive IgGs with estimated concentrations of 8 to 13 ng/ml (**Figure 5**). Interestingly, 10/333 (3.0%) primary school children had SARS-CoV-2 antibodies, with a median antibody concentration of 12.8 ng/ml (range 6.8 to 89 ng/ml).

The antibody prevalence ratio (PR) of primary school to preschool kids was 7.6 (95% CI: 1.9 – 30.6). Thus, the antibody prevalence was significantly elevated in primary school children compared to the preschool cohort ($p = 0.0047$, Fisher's exact test, two-tailed). Out of 12 children with identified salivary IgG during autumn 2020, only 1 child (7 years old) was aware of the infection with one of their household members being infected (**Table 5**). Additionally, 3 children self-reported potential exposure to a SARS-CoV-2 infected household member (2 reported a SARS-CoV-2 infection in a household member and additionally 1 reported loss of taste/smell) (**Table 5**). In addition, of the 12 IgG positives indicating a potential prior infection, only 2 (17%) self-reported cold-like symptoms in the preceding 2 months, whereas the majority (83%) did not report any symptoms.

The adult cohort of 383 individuals (0.5% of the adult population in Tübingen) – mostly accompanying parents and tutors - only allowed for limited insights into COVID-19 epidemiology. Estimated prevalence of SARS-CoV-2 antibodies

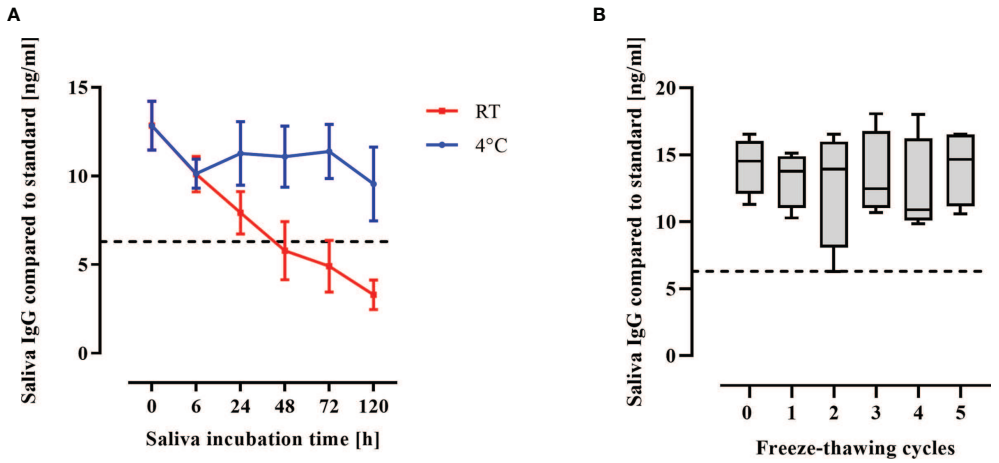


**FIGURE 4** | Antibody stability in saliva. **(A)** Saliva was kept at RT or at 4°C for respective durations (h) after collection and SARS-CoV-2 IgG concentrations were measured by saliva ELISA. Mean and standard error of the mean of 4 biological replicates is shown. **(B)** Collected saliva was subjected to freeze-thaw cycles and IgG concentrations were measured by saliva ELISA. IgG concentrations is shown as boxplots. Dashed line: Cutoff for antibody positivity.

in saliva was 0.9% (95%CI: 0.3%-2.6%) and only 3 (0.8%) persons had SARS-CoV-2 antibodies (6.9, 7.7, and 13.7 ng/ml) (**Table 4**). None of the IgG positive adults were aware of a previous infection, but 1/3 (0.3%) and 2/3 (0.7%) self-reported infection with a loss of taste and/or smell in household members (**Table 5**). From 1 of these 3 salivary IgG positive adults plasma as well as another saliva sample could be sampled 4 weeks later and IgG positivity could be confirmed with EUROIMMUN and with saliva ELISA, respectively. Self-reported SARS-CoV-2 infection was seen for 4/837 children and 6/383 adults (**Table 3**) amongst only 1 individual (child) was also found SARS-CoV-2 IgG positive by saliva ELISA (**Table 5**, participant no. 7).

Of the 9 participants (3 children and 6 adults) who self-reported a previous SARS-CoV-2 infection but were negative for salivary SARS-CoV-2 IgG, 3/9 reported an exposure to infected household members (of note: none of the self-reported SARS-CoV-2 infection was diagnosed by PCR (**Supplementary Table 4**).

## DISCUSSION

In response to the COVID-19 pandemic, we developed a saliva ELISA that facilitates population-based serological surveys for SARS-CoV-2 infections. Asymptomatic patients are often not routinely tested for SARS-CoV-2 infections, in particular the paediatric population, has been underrepresented in epidemiological studies (6). We report here the validation of the saliva ELISA using paired plasma and saliva samples of carefully characterized adults. We then applied the saliva ELISA to estimate exposure of young children to SARS-CoV-2 by early December 2020, during the rise of the second pandemic wave in Germany. The data presented here is part of an ongoing prospective, longitudinal study to follow SARS-CoV-2 antibody prevalence in children aged 1 to 18 years over 3 timepoints within 12 months (summer 2020 to summer 2021). None-invasive saliva sampling is done at respective educational institutions randomly located over the area of Tübingen, a University town in Germany. Estimates for prevalence of SARS-CoV-2 antibodies in young children aged 1 to 10 years was 1.6% and indicated about a 3-fold higher exposure to SARS-CoV-2 compared to the reported PCR diagnosed cases given by the local health department. In accordance with other observations (21, 22), prevalence in preschool children was significantly lower compared to primary school children. Antibody screening revealed the overall extent to which this older cohort had been exposed. Fascinatingly, only 16% of SARS-

**TABLE 3** | Characteristics of the study populations.

| | Preschool children | Primary school children | Preschool adults* | Primary school adults* |
|---|---|---|---|---|
| Saliva samples, n | 504 | 333 | 308 | 75 |
| Age, median in years (range) | 4 (1 – 6) | 9 (6-10) | 38 (19-62) | 46 (20-67) |
| Previous cold-like symptoms, n (%) | 244 (48) | 124 (37) | 102 (33) | 30 (40) |
| Previous SARS-CoV-2 infection, n (%) | 2 (0.4): 2c | 2 (0.6): 1b, 1c | 5 (1.6): 1b, 4c | 1 (1.3): b |
| Previous SARS-CoV-2 infection in household member, n (%) | 13 (2.6): 9b, 4c | 8 (2.4): 2a, 1b, 5c | 6 (1.9): 1a,1b, 4c | 2 (2.6): 1b, 1c |
| Loss of taste/smelt in household members, n (%) | 12 (2.4%) | 7 (2.1%) | 7 (2.3%) | 2 (2.6%) |

**Adults, accompanying parents and tutors; a, Reported a positive PCR within 6 months prior to sampling; b, Reported a positive ELISA within 6 months prior to sampling; c, No information provided if prior diagnosis was based on PCR or ELISA.*

**TABLE 4** | Salivary SARS-CoV-2 IgG prevalence and infections within the pandemic year 2020 in Tübingen city.

| | Saliva samples, n[§] | Salivary IgG positive, n* | Salivary IgG positivity rate, %* | Estimated salivary IgG prevalence (95% CI)* | | PCR positive, n[#](city) | PCR positive, %[#](city) |
|---|---|---|---|---|---|---|---|
| All children | 837 | 12 | 1.4 | 1.6 | (0.9-2.9) | 34 | 0.5 |
| Preschool children | 504 | 2 | 0.4 | 0.5 | (0.1-1.7) | 15 | 0.4 |
| Primary school children | 333 | 10 | 3.0 | 3.4 | (1.9-6.2) | 19 | 0.7 |
| All adults | 383 | 3 | 0.8 | 0.9 | (0.3-2.6) | 1,249 | 1.4 |
| Preschool adults | 308 | 3 | 1.0 | 1.1 | (0.4-3.2) | N/A | N/A |
| Primary school adults | 75 | 0 | 0 | – | – | N/A | N/A |

*[§]Saliva was sampled between 09 September 2020 and 02 December 2020. *SARS-CoV-2 IgG was detected by the saliva ELISA. [#]PCR confirmed SARS-CoV-2 cases represent the total cases in the city of Tübingen (not within the study cohorts) and were derived from the weekly reports for Tübingen city reported by the Gesundheitsamt Tübingen that can be obtained via https://www.kreis-tuebingen.de/17094149.html. Data for the children were provided by the Gesundheitsamt Tübingen. The cut-off date was 30 November 2020. Cases in % were either calculated for the total Tübingen population or the respective Tübingen children cohorts. N/A, Not available. Adults were accompanying parents and tutors.*

CoV-2 IgG positive children reported symptoms, while at least 84% of the positives were asymptomatic.

Saliva ELISA testing in the preschool kids and in adults did not identify more cases than known by official PCR records. Therefore, the results for adults should not be overinterpreted as only a small sample of 0.5% of the total adult population in the city was investigated. SARS-CoV-2 antibodies indicate that a person has been infected (and/or COVID-19 vaccinated) and IgGs can be detected in the blood as soon as two weeks after infection (23) and beyond. This is consistent with previous findings (24), where about two-thirds of the mild COVID-19 adult controls still had RBD binding IgG titres 6 months after symptom onset in blood - but also in saliva although at a lower magnitude. SARS-CoV-2 RBD antibodies possess virus neutralization activity but relevant titres and their role in protection from infections is still under investigations. It was interesting to see that COVID-19 vaccination induced SARS-CoV-2 IgG titres in saliva (and to a lesser extent also in IgA) and levels were similar to those found in blood (12, 13). Secreted IgA is produced by plasma cells of the mucosae and associated glands in the oral cavity, whereas IgG in saliva originates mainly from plasma cells residing in the bone marrow, circulating in the blood, before entering the saliva *via* transudation (25). As shown by others and confirmed by our work, SARS-CoV-2 IgG in saliva correlates well with the response in blood, therefore saliva IgG levels may therefore reflect the systemic antibody response. Saliva collection is a convenient alternative to blood sampling that allows for self-sampling and is easy to use in the paediatric population. This is especially important for studies that need to be conducted in non-medical settings without skilled personnel. Self-sampling devices include plastic containers for simple drooling or commercial devices that are built on saliva-absorbent tissues (e.g. Oracol, OraSure, etc.). The latter is particularly useful for the sampling of infants who often have

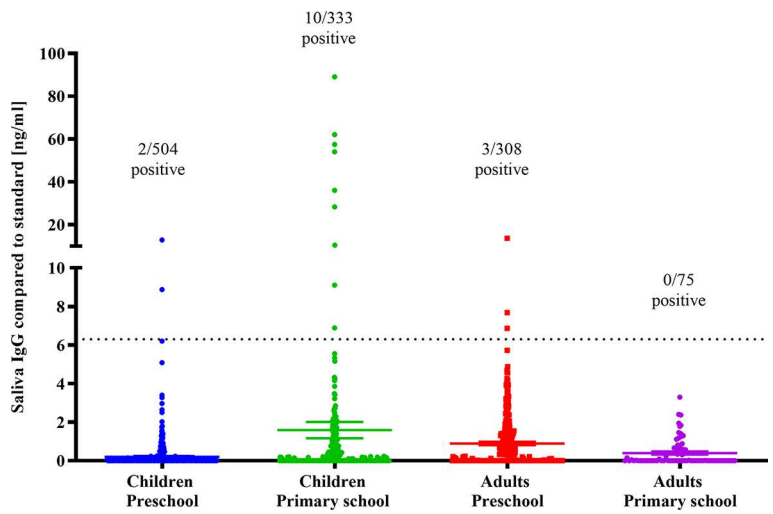


**FIGURE 5** | SARS-CoV-2 IgG in saliva of young children and adults. SARS-CoV-2 RBD reactive IgG concentrations in saliva of preschool children and primary school kids and accompanying parents and tutors (adults). Dotted line: Cutoff for SARS-CoV-2 IgG positivity.

**TABLE 5** | Characteristics of salivary SARS-CoV-2 IgG positive individuals.

| Participant | IgG titre[#] (ng/ml) | Age (years) | Cohort | Previous SARS-CoV-2 infection* | Symptoms in preceding 2 months* | Previous SARS-CoV-2 infection in household members* | Loss of taste/smell in household members* |
|---|---|---|---|---|---|---|---|
| 1 | 8.9 | 2 | pre-school | No | No | No | Yes |
| 2 | 12.9 | 6 | pre-school | No | sore throat | No | No |
| 3 | 36.1 | 7 | primary | No | No | No | No |
| 4 | 89.0 | 9 | primary | No | No | No | No |
| 5 | 28.3 | 9 | primary | No | No | No | No |
| 6 | 57.5 | 8 | primary | No | No | Yes | Yes |
| 7 | 62.0 | 7 | primary | Yes | No | Yes | Yes |
| 8 | 54.0 | 10 | primary | No | No | No | No |
| 9 | 9.1 | 9 | primary | No | cough, rhinitis | No | No |
| 10 | 6.9 | 9 | primary | No | No | No | No |
| 11 | 10.4 | 8 | primary | No | No | No | No |
| 12 | 10.4 | 8 | primary | No | No | No | No |
| 13 | 13.4 | 37 | adult | No | No | No | No |
| 14 | 7.7 | 40 | adult | No | No | Yes | Yes |
| 15 | 6.9 | 33 | adult | No | No | No | Yes |

[#]*Saliva IgG compared to standard [ng/ml].*

**Self-reported by a structured questionnaire on the day of sampling.*

difficulties with controlled spitting. However, the device should be carefully chosen and documented as the collection methodology might impact the antibodies observed. In addition, quantification of specific antibody using the saliva ELISA could be altered by the hydration status or the gum health of the individual, but sensitivity or specificity should not be affected by this.

As of May 2021, there is no ELISA commercially available that is approved for detection of salivary IgG reactive to SARS-CoV-2 RBD; one kit detects IgG binding to the nucleocapsid protein in oral fluids (ref 1-1260, Salimetrics, LLC) that is highly homologous to endemic coronaviruses. The published sensitivity and specificity of the test is similar to the saliva ELISA reported here (92% and 98%, respectively), but the slightly reduced specificity has a strong impact on the positive predictive value in diseases with low prevalence of salivary SARS-CoV-2 antibodies (13). Published studies on salivary SARS-CoV-2 IgG either used advanced immunoassay techniques [Luminex (11), immunoprecipitation systems (26)], in-house ELISAs, or repurposed commercial ELISAs for salivary IgG measurement (27, 28). All work reported a consistent positive correlation between blood and saliva antibodies particularly for RBD specific IgG. Nonetheless, due to the known lower concentration of antibodies in saliva compared to blood (29), all assays required protocol optimizations to increase sensitivity while controlling for an optimal signal-to-noise ratio. The saliva ELISA protocol reported here includes an improved blocking strategy and a simple biotin-avidin signal amplification step. The blocking condition for each of the ELISA steps were optimized, the final protocol uses a different antibody for the blocking of the antigen and the blocking of the secondary antibody. This is unusual but can be explained by the different nature of interaction between the antigen and the saliva/serum antibodies or the interaction of the human antibody and the secondary antibody. To create a saliva ELISA protocol for population-based surveys (and less for patient care), we tailored the test performance towards 100% specificity and accepted a lower sensitivity of approximately 87%. A population-based antibody survey, in the current low prevalent COVID-19 epidemiology, shows that seroprevalence estimates are much more affected by a low test specificity then by a low test sensitivity [e.g. if the 'true' COVID-19 prevalence is 5%, the estimated seroprevalence could double when the specificity is 'only' 95%, however estimates would decrease by max. 1% when the sensitivity is 'only' 80% (30)].

The sensitivity and specificity of the saliva ELISA was determined with stringently selected samples based on two different serum ELISA having the emergency use authorization (EUA) form the FDA. Discordant results from the two assays were excluded from the saliva ELISA validation, as it is not very clear how these samples should be classified. Regarding caveats and limitations, we are still unsure for how long SARS-CoV-2 IgG remain detectable in the saliva of children. For antibodies in the plasma and saliva of adults, titres can be detected for at least 6 months after infection as shown by our results and reports from the literature. Titres in saliva have been reported to be considerably lower compared to plasma and thus, any tests reach its detection limits. Despite little sequence homology of SARS-CoV-2 RBD to endemic coronaviruses (31), cross-reactivity of antibodies induced by seasonal human coronaviruses infections to the RBD saliva ELISA cannot be fully excluded although generally considered minimal (32–34). A study has shown that approximately 1% of prepandemic blood samples from children and adults cross-reacted with SARS-CoV-2 RBD (35), even though the comparison of different studies can be difficult as assay conditions play a pivotal role. In this line, specificity of the saliva ELISA using saliva from children sampled in prepandemic times would be of great value but were not available. Additionally, performance outcomes of the saliva ELISA always depend on the reference serology applied - here on EUROIMMUN sample classification and a different sensitivity or specificity cannot be excluded.

Saliva sampling was done in educational institutions but unless there was an obvious clustering of cases unfortunately, we are unable to provide information regarding the efficacy of school measures to combat the pandemics. It is also difficult to make informed statements in relation to an institution´s methods to limit the

spread of SARS-CoV-2, as the influence of constant measures such as school closures and other lockdowns like curfews have significantly reduced transmission rates. It will be of high interest to investigate the prevalence of SARS-CoV.2 antibodies in children and all vulnerable populations in other areas of Europe and around the world.

The saliva ELISA is a valid and suitable protocol to enable population-based surveys for SARS-CoV-2 antibodies, that are needed to monitor SARS-CoV-2 explorations in all age groups. Using non-invasive sampling and saliva ELISA testing, we were able to elucidate that the antibody prevalence in infants and children attending preschool facilities was significantly lower than children attending primary schools.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The study protocol was approved by the ethics committee of the University Hospital Tübingen (B312/2020BO1) and registered at Clinicaltrials.gov (NCT04581889). Written informed consent was obtained from adults or the child's parents or legal guardians if under the age of 18 years.

## AUTHOR CONTRIBUTIONS

AK, RF, JH, BM, and PK conceived and designed the study. RF, KE, and CH designed and conducted all experiments. YP and EF did the sample collection and sample processing. YP supervised the study conduct. CH, YP, JH, RF, and AK verified and analysed the data. JH, RF, and AK did the statistical analysis. AK, CH, YP, and RF wrote the first manuscript draft. AK, RF, and JH revised and finalized the manuscript. All authors reviewed and approved the contents of the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.753435/full#supplementary-material

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# SARS-CoV-2 Antibodies Are Persisting in Saliva for More Than 15 Months After Infection and Become Strongly Boosted After Vaccination

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SARS-CoV-2 antibodies in saliva serve as first line of defense against the virus. They are present in the mucosa, more precisely in saliva, after a recovered infection and also following vaccination. We report here the antibody persistence in plasma and in saliva up to 15 months after mild COVID-19. The IgG antibody response was measured every two months in 72 participants using an established and validated in-house ELISA assay. In addition, the virus inhibitory activity of plasma antibodies was assessed in a surrogate virus neutralization test before and after vaccination. SARS-CoV-2-specific antibody concentrations remained stable in plasma and saliva and the response was strongly boosted after one dose COVID-19 vaccination.

**Keywords: SARS-CoV-2, COVID-19, antibody, saliva, mucosa, convalescent, vaccination, neutralization**

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## INTRODUCTION

The COVID-19 pandemic has now been ongoing since December 2019 and has received major social, political, and scientific attention from the beginning. Both SARS-CoV-2 infection and COVID-19 vaccination elicit an immune response that largely protects from (re)infection and severe disease (1). Although immune correlates of protection have not yet been pinned down, antibody-mediated immunity is a critical constituent (2, 3). The SARS-CoV-2 specific humoral immune response characteristics, including magnitude, longevity and function have been - and still are - classically studied for serum immunoglobulins. So far, little attention has been paid to the presence of SARS-CoV-2 antibodies in the mucosa of the upper respiratory tract, the primary entry route for the virus (4). An established model for the investigation of the role of mucosal immunity against oral infection is the murine cytomegalovirus (5), in which a specific vaccination elicits high antibody titers in salivary glands that are leading to the protection from an otherwise deadly challenge (6). This mechanism has also been proposed for other respiratory viruses such as the influenza A virus (7, 8). Evidence is accumulating that SARS-CoV-2 spike protein reactive

antibodies in respiratory mucosa can also control virus replication (9). In a nonhuman primate infection model, it was shown that mRNA-1273 (Moderna) vaccinated animals with higher spike protein-specific IgG in the mucosa were less likely to be infected after challenge, and naïve hamsters were protected by passive transfer of vaccine-induced IgG (10). SARS-CoV-2 antibodies are present in the mucosa, more precisely in saliva, after a recovered infection and the following vaccination for at least 9 months (11–14).

To shed light on the association between antibodies in plasma and in mucosa, we compared the kinetics of the SARS-CoV-2 spike protein IgG concentrations over a 15-month follow-up period after recovery from mild COVID-19 disease. We further investigated if the antibody response deriving from the SARS-CoV-2 infection is boosted following one dose of a commercially available COVID-19 vaccine that could be able to control SARS-CoV-2 infection and transmission.

# METHODS

## Study Design

Between May and October 2020 adults who had a SARS-CoV-2 infection had been externally diagnosed by qPCR (n = 40) or ELISA (n = 32) and documented by a medical certificate were included in the study. The reported date of symptom onset (PSO) served as a surrogate date for SARS-CoV-2 infection. Duration of disease and symptoms were collected *via* a questionnaire.

## Sample Collection and Processing

Sample processing and storage was done as published recently (12). Briefly, saliva was collected by spitting into a sterile container (3ml) (Multi-purpose containers 30ml Greiner Bio-One ref. 201150) and kept at 4°C with a maximum of 6 hours until sample processing. The tubes were centrifuged at 2500 rpm for 6 min and the supernatant was inactivated using tri-n-butyl phosphate (TnBP) 0.3% and Triton X-100 1% at final concentrations. Samples were stored at -20°C for the further analysis. Peripheral blood was collected using 9 ml lithium heparin monovettes. Plasma was isolated and immediately collected by centrifugation at 1400 rpm for 10 min and stored at -20°C until use.

## ELISA for SARS-CoV-2 IgG Detection in Saliva and Plasma

IgG reactive to SARS-CoV-2 RBD were measured by a meticulously established and validated in-house ELISA (12, 13) for IgG detection in saliva as well as by an in-house ELISA specifically established to analyze plasma antibodies (see **Supplementary Material**). Briefly, the SARS-CoV-2 RBD antigen was diluted in 1x PBS to a final concentration of 2 µg/ml and added per well to high binding plates. After overnight incubation, the wells were washed and blocked with The Blocking Solution for 2 hours at room temperature (RT) on a microplate shaker (700 rpm). Subsequent washing steps were repeated. Saliva and control samples were serially diluted from 1:3 up to 1:19,683 using The Blocking Solution. 100 µl sample dilution was added per well and incubated for 1 hour (RT, 700 rpm). IgG antibody presence was detected by 1:20,000 diluted biotinylated anti-human IgG and 1:1,000 Avidin-HRP. For visualization, 100 µl TMB substrate solution was added, and the reaction was stopped using 1 M HCl. The plate was read at 450 nm and 620 nm with a microplate reader (CLARIOstar, BMG LABTECH). Cut-off for salivary SARS-CoV-2 IgG positivity was previously set to 6.3 ng/ml (12).

The SARS-CoV-2 IgG detection in plasma was performed using an established in-house assay following similar parameters used in the saliva ELISA assay, with some exceptions: Plasma was diluted 1:100 up to 1:7,812,500 dilution in The Blocking solution (1 to 5 dilution row) and for IgG detection an HRP coupled anti-human IgG was used (ref. 109-036-097, Jackson Immuno Research Laboratories). The detection antibody was diluted 1:5,000 in 1x ROTI Block buffer and incubated for 30 min (RT, 700 rpm). The IgG concentration is presented in µg/ml.and the cut-of value was set to 4.0 µg/ml (12). For confirmation, plasma samples were re-analyzed by the commercial, CE certified SARS-CoV-2 IgG ELISA (EUROIMMUN) detecting antibodies binding to SARS-CoV-2 Spike protein domain S1 and the assays were performed following the manufacturer's instructions.

## Surrogated Virus Neutralization Assay

A surrogate virus neutralization test (SARS-CoV-2 NeutraLISA, ref. EI2606-9601-4 kindly provided by EUROIMMUN) was performed following manufacturer's instruction to estimate the inhibitory activity of SARS-CoV-2 IgG in plasma in a non-BSL-3 set-up (see **Supplementary Material** for detailed description of the assay procedures).

## Data Analysis

RStudio (Version 1.2.5001), running R (version 4.0.4.) and Graph Pad Prism (Version 9.1) were used for descriptive statistical analyses. The data was analyzed at 95% confidence interval ($p<0.05$). Linear regression modelling was performed to assess change of IgG concentrations over time, and local polynomial regression was used to model the kinetics of antibody levels before and after vaccination using the stats package (version 3.6.2) in R. Pairwise Pearson's product-moment correlation coefficient was applied to assess the correlation of IgG concentration in saliva and in plasma as well as between % neutralization and plasma IgG concentrations. Non parametric Wilcoxon rank sum paired test was performed to check for significant difference of IgG concentrations or of % neutralization before and after vaccination.

For all statistical analyses, significance level (alpha) was set to 0.05.

# RESULTS

Between May and October 2020, 72 adults (male/female; n = 25/47, median (range) age in years = 29 (19–75)) who had suffered

from mild COVID-19 (all participants not hospitalized, loss of smell and taste: n = 50) were enrolled to monitor and longitudinally compare the kinetics of SARS-CoV-2 reactive IgG in saliva and in plasma after convalescence. Additionally, the virus inhibitory activity of plasma antibodies in a surrogate virus neutralization test was assessed.

Approximately 4 months (median: 132 days, range: 42-171 days) post COVID-19 symptom onset (PSO), first sampling was done, and 89% (64/72) participants were positive for SARS-CoV-2 RBD specific IgG in plasma as analyzed by the established in-house ELISA and confirmed by commercial EUROIMMUN ELISA (58/72 were IgG positive and 5/72 were IgG intermediate) (**Supplementary Figures 1A, B**). Participants were followed for 5 additional bimonthly timepoints, at which saliva and plasma were collected, covering a total period of in average 15 months after SARS-CoV-2 infection (median PSO: 447 days, range: 367–497 days). During the follow-up period, none reported a re-infection, but 58% (42/72) participants received COVID-19 vaccination (22 Corminaty/BioNTech, 7 Spikevax/Moderna, 13 Vaxzevria/AstraZeneca) at any time between 235 and 409 days PSO.

Half a year after infection (median PSO: 196 days, range: 104-226 days), SARS-CoV-2 IgG were present in plasma of 89% (64/72) of volunteers or in saliva of 72% (46/72) (all saliva IgG positives were also positive for plasma IgG). At the end of the follow-up period, either 15 months PSO or at the last follow-up before vaccination, still 98% (63/64) and 80% (37/46) had SARS-CoV-2 reactive IgG in plasma and in saliva, respectively. IgG concentrations in plasma (**Figure 1A**) and in saliva (**Figure 1B**) remained constant during this follow-up period of up to 15 months (flat regression lines). IgG concentrations in paired saliva and plasma samples

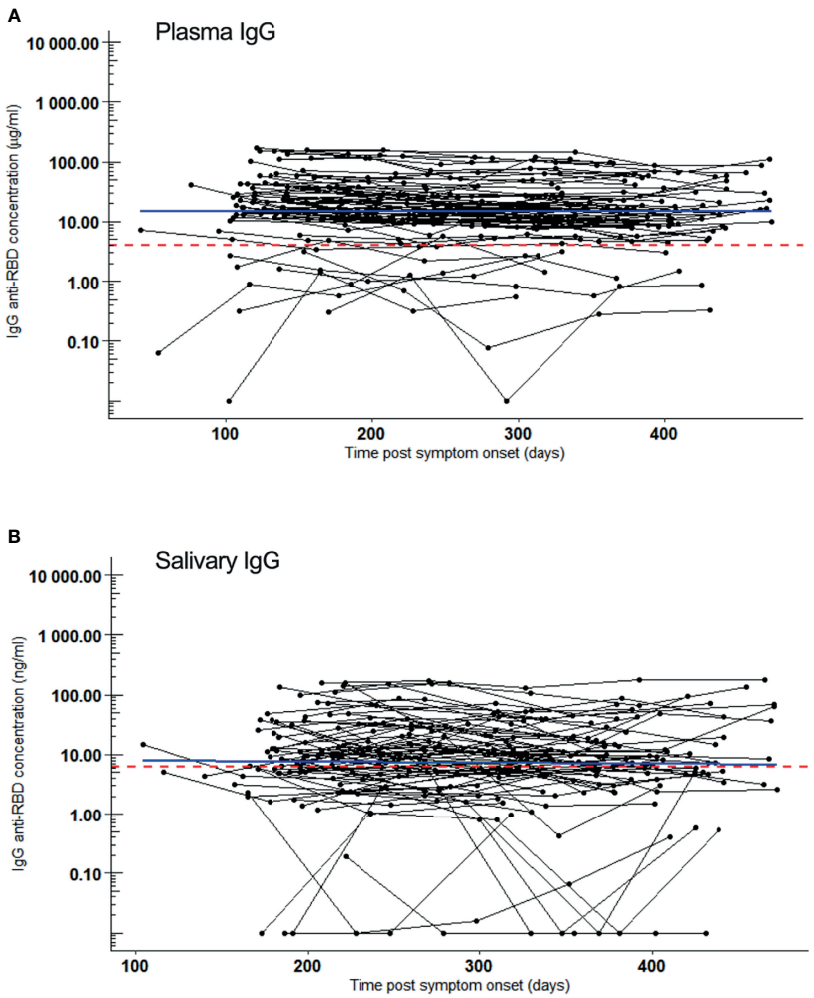


**FIGURE 1** | SARS-CoV-2 RBD reactive IgG over a 15-month follow-up period. Samples were collected 5 times, bimonthly from 72 convalesced volunteers who had mild COVID-19. IgG kinetics are shown per individual (black line). Sampling time points (black dots) are given as days post symptom onset (PSO). Blue line: regression line. Red dotted line: Cut-off for positivity. **(A)** IgG (µg/ml) kinetics in plasma after COVID-19 convalescence. Slope of linear regression = -0.02, p = 0.2. **(B)** IgG (ng/ml) kinetics in saliva after COVID-19 convalescence. Slope of linear regression = 0.009, p = 0.7.

were highly correlating (Pearson's product-moment correlation r = 0.73, [95% confidence interval (CI): 0.67-0.78)] (**Supplementary Figure 2**). Interstingly, the median IgG concentrations in saliva were 1,206-fold (95% CI: 1,062-fold to 1,493-fold) lower than in plasma.

COVID-19 vaccination induced a remarkable and significant increase of SARS-CoV-2 RBD reactive IgG concentrations - both in plasma and in saliva of COVID-19 convalescent volunteers (**Figures 2A, B**). IgG concentrations increased 72-fold in plasma ($p = 7.2x10^{-12}$) and 46-fold in saliva ($p = 6.9x10^{-11}$) within one month (median: 26 days) after first vaccination when compared to those before vaccination (median: -39 days), reaching median concentrations of 1458 µg/ml and 626 ng/ml, respectively. Volunteers negative for SARS-CoV-2 IgG in plasma or saliva before vaccination also increased significantly in antibody levels in plasma and saliva (median IgG plasma 449 µg/ml and 137 ng/ml) after vaccination. Although, these concentrations were remarkably lower to those reached by volunteers positive for IgG prior to vaccination (3.3 fold for plasma IgG and 4.6 fold for salivary IgG), this difference did not reach statistical significance ($p = 0.21$ and $p = 0.10$ for plasma and saliva, respectively).

To assess the plasma for virus neutralizing antibodies, an ACE2 receptor/antibody competition assay was done as surrogate measure for virus neutralization of those individuals with plasma IgG. At the first sampling (4 months PSO), 34% (17/50) individuals had neutralizing antibodies and this fraction did not largely change until 8.5 months later [median: 254 days, range 111 – 334 days; 27% (14/50)]. Of this cohort, 33 became

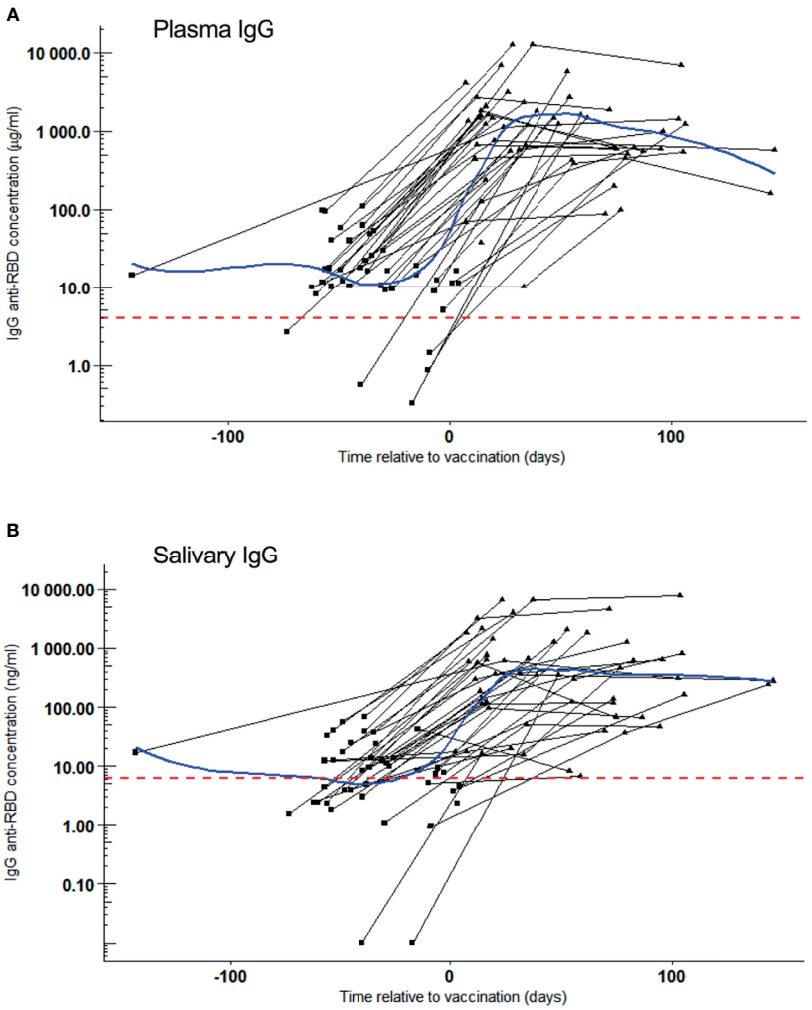


**FIGURE 2** | SARS-CoV-2 RBD reactive IgG after vaccination. Samples were collected of whom 42 became vaccinated during the study. IgG kinetics are shown per individual (black line). Sampling time points (black dots) are given as days post symptom onset (PSO). Blue line: Local Polynomial Regression. Red dotted line: Cut-off for positivity. **(A)** Plasma IgG (µg/ml) concentration per convalesced volunteer before (square) and after (triangle) one dose of COVID-19 vaccination. Day 0 represents the reported day of vaccination. Local Polynomial Regression, residual standard error: 1979. **(B)** Salivary IgG (ng/ml) concentration per convalescent volunteer before (square) and after (triangle) one dose of COVID-19 vaccination. Day 0 represents the reported day of vaccination. Local Polynomial Regression, residual standard error: 1354.

vaccinated 2 months later (median: 70 days, range: 55 – 167 days) and 100% (33/33) attained high neutralization activity (**Figure 3A**). Neutralization activity in plasma of vaccinated participants was significantly different from those who remained unvaccinated (median inhibition of 96% versus 10%, respectively, $p = 3.3x10^{-8}$). Notably, SARS-CoV-2 RBD IgG concentrations in plasma correlated positively with neutralization activity [Pearson correlation, r=0.70 (**Figure 3B**)].

## DISCUSSION

SARS-CoV-2 immunity in the respiratory mucosa, particularly in saliva, includes locally secreted dimeric IgA as well as IgG that comes largely from the systemic immune response *via* gingival transduction in the gums (15). Correlating IgG plasma and saliva levels from recovered COVID-19 patients have been reported to persist for at least 3 months (15–17). Our study provides evidence of long-term and constant SARS-CoV-2 RBD IgG levels for most COVID-19 convalescent individuals not only in plasma but - to our knowledge - for more than one year in saliva. In the absence of a defined correlate of protection, SARS-CoV2 RBD IgG levels serve as a proxy for monitoring immune response. Here, one vaccination dose strongly boosted IgG levels not only in plasma but also in saliva. SARS-CoV-2 is transmitted orally and virus spike protein binding to the host angiotensin converting enzyme 2 (ACE2) of epithelial cells in the respiratory pathways permits virus infection and replication. If mucosal IgG elicited by a past SARS-CoV-2 infection and/or by vaccination can prevent (re)infection, replication and

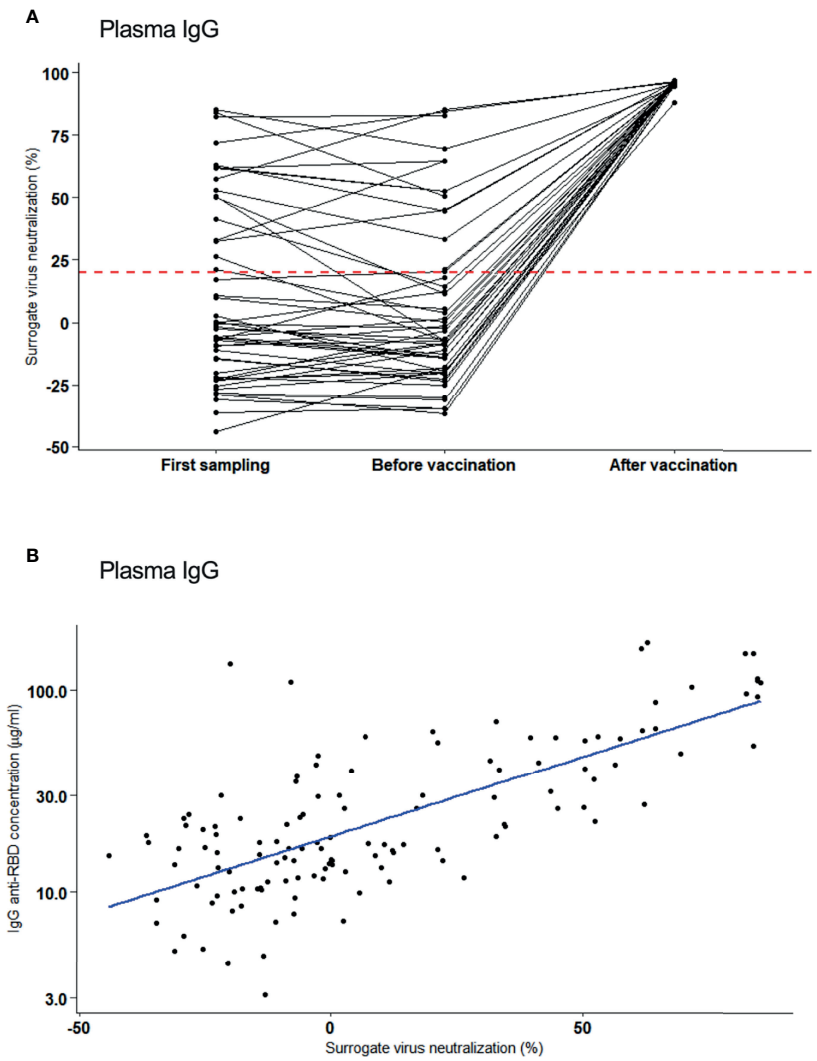


**FIGURE 3** | Neutralizing activity of plasma antibodies to SARS-CoV-2. **(A)** Inhibitory activity (%) (surrogate for virus neutralization) of plasma IgG assessed at first sampling (4 months after infection), before vaccination and after vaccination. Neutralization activity are shown per individual (black line). Red dotted line: Cut-off for positivity. **(B)** Correlation of neutralization activity (%) and plasma IgG concentration after one vaccination (Pearson correlation, r=0.70).

transmission, e.g. by interfering with spike protein binding, still needs to identified and quantified (9). Encouragingly, supporting findings come from a recent study in which vaccinated non-human primates with strong mucosal antibody responses were protected from an infection after SARS-CoV-2 challenge, revealing for the first time antibody concentration as a direct mechanistic correlate of protection (10). To gain functional insights into SARS-CoV-2 RBD IgG, a surrogate virus neutralization test was done, but for technical reasons, this was unfortunately only possible for plasma IgG due to low IgG levels in saliva. Remarkably, spike protein binding to ACE2 was only moderately inhibited by IgG in COVID-19 convalescent donors and was strongly increased after already one dose of any of the given vaccine. Future investigations should address the cross-neutralization against circulating SARS-CoV-2 variants of concern, e.g. the currently circulating Delta B.1.617.2 variant. In addition, assays capable of quantifying IgA-levels in saliva are needed, as these might also contribute to protection against SARS-CoV-2 infection.

In summary, evidence towards a role of mucosal antibodies in prevention of SARS-CoV-2 infection is accumulating.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article and the **Supplementary Material**. Further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University Hospital Tübingen (B312/2020BO1 and 247/2020BO1) and registered at Clinicaltrials.gov (NCT04581889) (18). All participants gave written informed consent to participate in the study.

## AUTHOR CONTRIBUTIONS

AK, RF, JH, ME, and PK conceived and designed the study. RF and CH designed and conducted all experiments. YP and L-FC did the sample collection and sample processing. YP supervised the study conduct. CH, YP, JH, RF, and AK verified and analysed the data. JH, RF, AK, and DC did the statistical analysis. AK, CH, YP, and RF wrote the first manuscript draft. AK, RF, and JH revised and finalized the manuscript. All authors reviewed and approved the contents of the manuscript. All authors confirm that they had full access to all the data in the study and accept responsibility to submit for publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.798859/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESEARCH LETTER

## Saliva and Plasma Antibody Levels in Children and Adolescents After Primary Infection With Omicron Variants of SARS-CoV-2 Infection in Germany

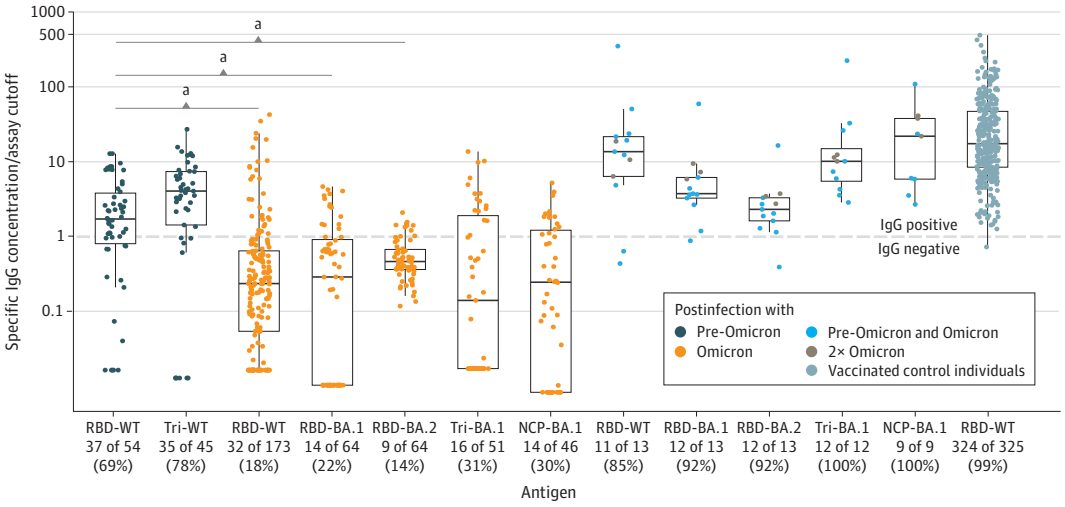
Omicron is escaping neutralization by antibodies induced by prior infections and vaccination.[1] Little information is available on antibody prevalence attributable to a primary Omicron infection; a challenging investigation as many individuals are already vaccinated and/or have had pre-Omicron SARS-CoV-2 infections. Antibodies in saliva may contribute to protection against recurrent SARS-CoV-2 infection.[2] Since summer 2020, we have been monitoring 1850 children and adolescents for exposure to SARS-CoV-2 in Tübingen, Germany, to investigate antibody response after primary Omicron infection on a large scale.

Supplemental content

**Methods** | This cross-sectional study is part of the longitudinal prospective observational study approved by the ethics committee of the University Hospital Tübingen.[3] This report follows the STROBE reporting guideline. Participants or their legal representatives gave written informed consent. Individuals with a prior SARS-CoV-2 infection as documented by a certified laboratory test (polymerase chain reaction or antigen) were included. Enzyme-linked immunosorbent assays were performed to quantify IgG reactive to SARS-CoV-2 receptor binding domain of the index (RBD-WT) and Omicron variants (RBD-BA.1, RBD-BA.2), to the spike trimer of the index (Tri-WT) and Omicron (Tri-BA.1), and to nucleocapsid-protein (NCP-BA.1) in saliva[4] and in plasma[5,6] (eMethods in Supplement 1).

**Results** | Saliva was collected from youth aged 1 to 18 years in spring 2021 (n = 1474) and spring 2022 (n = 1194) (groups not exclusive). Of these, 54 participants were infected with SARS-CoV-2 between April 2020 and December 2021 (defined as pre-Omicron infection), and 173 were infected from January 2022 to May 2022 (defined as Omicron infection). Either no symptoms or mild coldlike symptoms were reported, and none of the participants were hospitalized for COVID-19. While 37 of 54 youth with pre-Omicron infection had RBD-WT reactive IgG in their saliva, considerably fewer (32 of 173) were IgG positive after an Omicron infection (**Figure 1**). This proportion did not increase when assessing IgG reactivity toward RBD-BA.1 (14 of 64) or RBD-BA.2 (9 of 64). All participants who experienced 2 infections were positive for salivary IgG (reactive to RBD-WT = 11 of 13; BA.1 = 12 of 13; BA.2 = 12 of 13) and at levels higher than after a single Omicron infection. Almost all vaccinated participants (324 of 325) were positive for anti-RBD-WT IgG and had the highest IgG levels. Thirty-five of 45 participants with pre-Omicron infection had IgG reactive to Tri-WT in saliva, while only 14 and 16 of 51 with Omicron infection had IgG against Tri-WT and Tri-BA.1, respectively. Again,

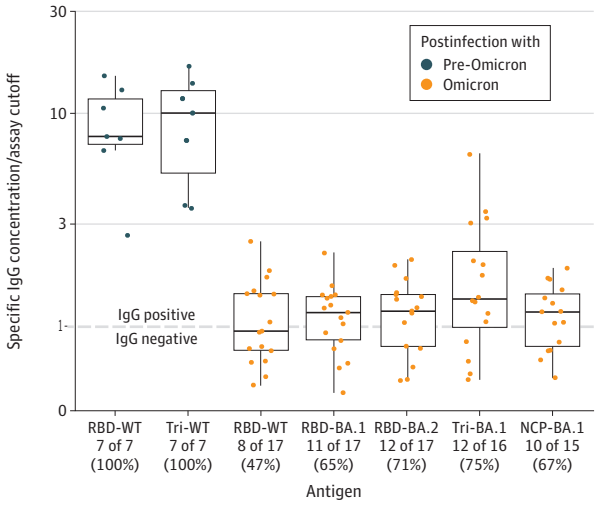
Figure 1. Saliva IgG Levels in Children and Adolescents Following SARS-CoV-2 Infection




Each dot represents 1 study participant and shows the ratio of the sample IgG concentration divided by the respective enzyme-linked immunosorbent assay cutoff for IgG positivity. Box plots show the distribution of the individual IgG ratios with median and 25th and 75th percentiles. Numbers and percentages indicate the percentage of IgG-positive samples among all samples measured.

[a] Significant at $P < .001$ by Wilcoxon rank sum test.

Figure 2. Plasma IgG Levels in Children and Adolescents Following SARS-CoV-2 Infection




Each dot represents 1 study participant and shows the ratio of the sample IgG concentration divided by the respective enzyme-linked immunosorbent assay cutoff for IgG positivity. Box plots show the distribution of the individual IgG ratios with median and 25th and 75th percentiles. Numbers and percentages indicate the percentage of IgG-positive samples among all samples measured.

individuals who were twice infected had salivary IgG reactive to spike trimer at relatively high levels.

For a subgroup of participants, plasma was sampled in addition to saliva. All participants with pre-Omicron infection had IgG reactive to RBD-WT and to Tri-BA.1 at high levels. For the Omicron-infected cohort, IgG reactive to RBD-WT, to RBD-BA.1, and to RBD-BA.2 and to the spike protein complex (Tri-BA.1) reflected the salivary IgG levels: only a fraction were IgG positive and IgG levels were low (**Figure 2**). Antibody levels reactive to NCP-BA.1 showed a similar pattern and consolidated spike protein–based findings (Figures 1 and 2).

**Discussion** | SARS-CoV-2 Omicron reinfections within short periods are increasingly reported.[7] Our data suggest that this might be explained by a weak humoral immune response to Omicron. In contrast to pre-Omicron variants, a primary Omicron infection elicited only a poor IgG antibody response, possibly resulting in insufficient infection prevention by salivary IgG. The observed neutralization escape of recurrent Omicron variants may be due to the absence of antibodies rather than reduced binding affinity. As a result, first-time SARS-CoV-2 infections with the Omicron variant may remain also largely undetected by seroprevalence studies. Further work on cellular immune responses and viral loads is needed to draw conclusions on the immunogenicity of Omicron and to test whether the results of this study are generalizable a larger population, including adults.

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*Acquisition, analysis, or interpretation of data*: Heinzel, Pinilla, Binder, Held, Fendel, Kreidenweiss.
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