Detection of COVID-19 infection in exhaled breath by gas chromatography coupled ion mobility spectrometry (GC-IMS)

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DETECTION OF COVID-19 INFECTION IN EXHALED BREATH BY GAS CHROMATOGRAPHY COUPLED ION MOBILITY SPECTROMETRY (GC-IMS)

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LIST OF ABBREVIATIONS

ACE-2 – angiotensin-converting enzyme 2

ARDS – acute respiratory distress syndrome

CLIA – chemiluminescence immunoassay

CoVs – coronaviruses

COVID –19 – coronavirus disease 2019

Ct – cycle threshold

CFR – caste fatality rate

CCL2/MCP1 – chemokine (C-C motif) ligand 2/ monocyte chemoattractant protein 1

CRP – C- reactive protein

CXCL1 – CXC Motif Chemokine Ligand 1

CXCL5 – CXC Motif Chemokine Ligand 5

ELISA – enzyme-linked immunosorbent assay

ESR – erythrocyte sedimentation rate

GC-MS – gas chromatography coupled ion mobility spectrometry

GM-CSF – granulocyte macrophage colony-stimulating factor

IL-6 – interleukin 6

IFA – immunofluorescence assay

IP-10 – interferon-gamma induced protein 10 kD

JAK – Janus kinase

LDH – lactate dehydrogenase

LFIA – lateral flow immunoassay

MERS- CoV – middle east respiratory syndrome coronavirus

MIP-1α – macrophage inflammatory protein 1-α

NIH – National Institutes of Health

PHEIC – public health emergency of international concern

RT-PCR – reverse transcriptase polymerase chain reaction

SARS- CoV – severe acute respiratory syndrome- coronavirus

SARS- CoV 2 – severe acute respiratory syndrome- coronavirus 2

RBD – receptor binding domain

RKI – Robert Koch Institute

TMPRSS2 – transmembrane serine protease 2

TNF-α – tumor necrosis factor -α

VOI – variant of interest

VOC – variant of concern

VOC – volatile organic compounds

WHO – World Health Organization

1 INTRODUCTION

1.1 Coronavirus disease 2019 (COVID 19)

1.1.1 Epidemiology

The recent COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, had an enormous impact on global health, economics, societies, and individual well-being. Its rapid global expansion emphasizes the need for a quick, noninvasive diagnostic tool as an example for faster management of viral communicable diseases. This brief introduction provides an overview of the epidemiology of COVID-19 and its situation in Germany.

In Wuhan, Hubei Province, China in late December 2019, a cluster of persons linked to the seafood and wet animal wholesale market were admitted to hospitals with pneumonia-like disease of unknown etiology (1). Symptoms included fever, dyspnea, cough, and pulmonary infiltrates on chest radiographs (2).

These were the first cases, in which a new variant of ß-coronavirus was detected. 2019 novel Coronavirus (2019-nCoV, later named SARS-CoV-2) spread rapidly through international travel, not only in China but to various countries around the world (3). Therefore, the World Health Organization (WHO) declared the coronavirus disease 2019 (COVID-19) outbreak a global pandemic on March 11, 2020 (4).

In response, governments and public health authorities worldwide have implemented various measures to limit the spread of the virus, stabilize and flatten the curve of hospitalizations in intensive care units, and regulate resources. These measures have included mandates for masks, restrictions on travel, social distancing and quarantine, lockdown and domestic movement limitations, and vaccination campaigns (5).

Germany reported its first confirmed case of COVID-19 on 28 January 2020, and from March 13, 2020, German states introduced lockdown measures. To contain the virus's spread, widespread testing, contact tracing and social distancing guidelines followed. The Robert Koch Institute (RKI), Germany's federal agency that manages disease control and prevention, played a pivotal role in monitoring the situation and providing recommendations (6). As of July 26, 2023, there were 38.4 million confirmed COVID-19 cases in Germany and 174.352 deaths related to the virus. This corresponds to an to an overall infection rate of 46.2% and a total mortality rate of 0.5%, based on a population of 83.1 million inhabitants. (7).

Due to declining virulence and increasing immunity, on 5 May 2023, after almost three years of pandemic, the WHO declared that COVID-19 had evolved into a persistent health challenge and was no longer classified as a public health emergency of international concern (PHEIC) (8).

Now present on every continent and in almost every country, SARS-CoV-2 has caused more than 768.9 million confirmed cases, including 6.9 million deaths (9), leading to an overall case fatality rate (CFR) of 0.9%. According to WHO, CFR may vary from 0.1-25%, depending on the country and region (10).

1.1.1 Coronaviruses and SARS-CoV-2

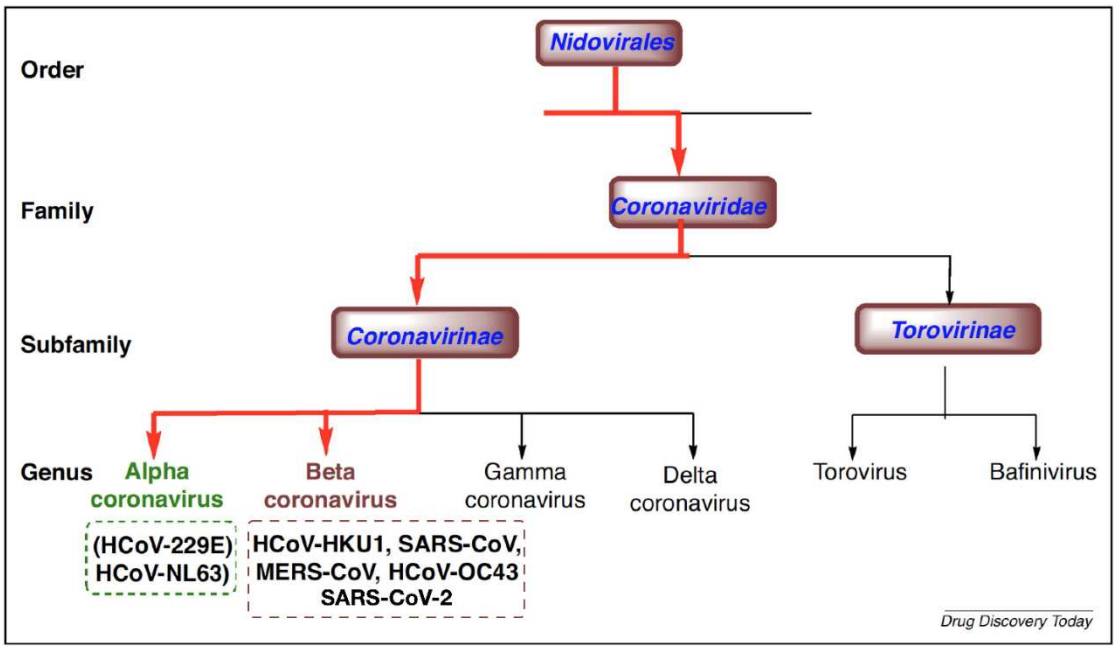

Figure 1. Taxonomy of Coronaviridae showing the alpha and beta coronaviruses that infect humans, including SARS-CoV-2 (11, 12)

Coronaviruses (CoVs) are characterized by being related, enveloped, positive-sense, single-stranded RNA- viruses. They belong to the family of Coronaviridae and can be further subclassified into four genera: Alpha- (α), Beta- (β), Gamma- (γ), and Deltacoronaviruses (δ). Coronavirus can infect a variety of domestic and wild animals, as well as humans, causing respiratory, gastrointestinal, hepatic, and neurologic diseases (13). From the CoVs, there have been 6 strains known to infect humans: 2 α-CoVs (229E and NL63) and 4 β-CoVs (HKU1, OC43, severe acute respiratory syndrome (SARS)-CoV, and Middle East respiratory syndrome (MERS)-CoV). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing the pandemic of COVID 19, is classified as the newest Betacoronavirus and the 7th strain, infecting humans.(Figure 1) (14).

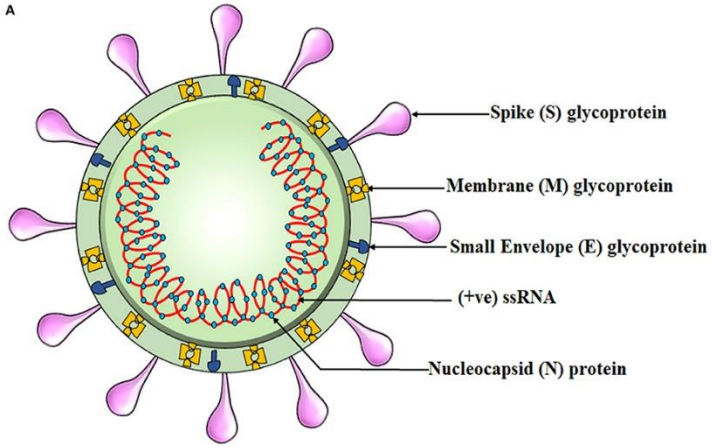

Figure 2: Schematic representation of the coronavirus structure (15)

Like SARS-CoV and MERS-CoV, SARS-CoV-2 possesses spike proteins on its envelope, which, gives it a typical crown-shaped appearance under the transmission electron microscope (Latin *corona*: wreath, crown) and therefore its name (16). Furthermore, the spike protein is for SARS-CoV-2 of crucial importance, as it facilitates viral entry by binding to ACE 2- receptors on the host surface (17). Despite the spike protein (S), SARS-CoV-2 encodes also for membrane, structural, and nucleoside proteins. The envelope (E) and membrane (M) proteins contribute to the structure of the viral envelope, while the nucleocapsid (N) protein facilitates the appropriate folding of genomic RNA into the nucleocapsid (18) (Figure 2). Additionally, the viral genome encodes 16 nonstructural proteins, which are involved in forming the replicase-transcriptase complex essential for viral replication (19).

There are 4 human CoVs, 229E, OC43, NL63, and HKU1, that have been circulating in the human population for decades, only causing mild respiratory symptoms for immunocompetent (20). On the other hand, SARS- CoV, MERS-CoV, and SARS-CoV-2 can cause pneumonia and even lead to death (21). These new CoVs have emerged in the last two decades through zoonotic transmission from other mammalian species, serving as their natural reservoir. As for SARS-CoV and MERS-CoV, bats possibly be the natural reservoir for SARS-CoV-2, as its genome is highly similar to that of the bat coronavirus (Bat CoV RaTG13), with a sequence identity of 96.2% (20, 22). The virus was then transmitted to humans through an intermediate host- the masked palm civet for SARS-CoV and the dromedary camel for MERS-CoV (23). For SARS- CoV-2 however, no intermediate host has been proven yet, but might have been present at the seafood market in Wuhan. Other research proposes that SARS-CoV-2

has been circulating for a much longer period within humans endemically, before its pathologic appearance in December 2019 (24, 25).

1.1.2 Transmission

Person- to person transmission of SARS- CoV-2 mainly occurs through respiratory fluids either in the form of inhaled droplets or air-borne particles.

Respiratory droplets are produced during speaking, laughing, sneezing as well as singing, having a size of ≥ 5-10 μm. Within 6 feet or 2 meters, respiratory droplets can directly reach another person's respiratory or nasal mucosa. Air borne articles or aerosols on the other hand are droplets of less than 5 μm that can remain airborne for a prolonged period. In areas with inadequate ventilation, the probability of transmission through airborne particles may be increased because the particles can remain in the air for minutes to hours, exposing people in proximity.

Additionally, besides droplets and aerosols as the primary mode of transmission, direct contact between mucous membranes (such as the eyes, nose, or mouth) and respiratory droplets from an infected individual can also result in transmission (26).

Transmission via contaminated surfaces (fomite transmission), although less important than respiratory transmission, should also be noted (27).

Vertical transmission of SARS-CoV-2 is possible but appears relatively infrequent. A systematic review and meta-analysis conducted by Kotlyar et al. revealed that in instances where 3rd-trimester pregnant women tested positive for COVID-19, the virus was transmitted to the fetus or newborn in only 3.2% of the 936 neonates tested (28).

1.1.3 Pathogenesis and pathophysiology

The route taken by SARS-CoV2 to reach the lungs is via the naso-oral cavity (15). Once the virus is inhaled, it enters the epithelial cells of the nasal cavity by binding to angiotensin-converting enzyme-2 (ACE-2) receptor with its spike protein (S) (29). As a key viral membrane antigen, the S protein exerts importance not only for the virus but also the host, as its recognition stimulates the production of a substantial number of neutralizing antibodies within the host (24). The spike protein consists of two main regions, S1 and S2 namely. S1, comprising the receptor binding domain (RBD), is responsible for binding host ACE-2- receptor, whereas S2 leads to membrane fusion (30). ACE-2- receptors are mainly found on respiratory epithelial cells, but also in the cornea, heart, kidney, bladder, gastrointestinal tract, on vascular endothelium and in

the placenta/decidua, implicating underlying mechanism of broad spectrum involvement (31, 32), implicating underlying mechanism of broad spectrum organ involvement.

After successful binding to the ACE-2 receptor, the viral spike protein undergoes cleavage by the transmembrane serine protease 2 (TMPRSS2), which is considered an essential step in effecting virus infection (29). It is noteworthy that nasal secretory and ciliated cells express the highest concentration of ACE-2 receptors and TMPRSS2. This observation elucidates the pivotal role played by these cells as the sites of initial infection, replication, and dissemination within and between individuals (31). In the lungs, it was discovered that bronchial transient secretory cells have the highest co-expression of the ACE2 receptor and TMPRSS2 (33). Additionally, the esophagus, ileum, colon, and superficial conjunctival cells all co-express both genes, which may explain the involvement of the gastrointestinal and ocular systems in cases of COVID-19 (31).

Cleavage of the spike protein by TMPRSS2 leads to conformational changes, which activate viral with host cell membrane fusion, permitting viral entry. Cathepsin B/L, another protease, provides an alternative way for viral entry. After viral endocytosis, it facilitates viral and endosomal membrane fusion (34) (Figure 3).

The virus is subsequently replicated in the host cell and packed into mature virions, which invade adjacent cells or are expelled via respiratory droplets or aerosols to infect new hosts.

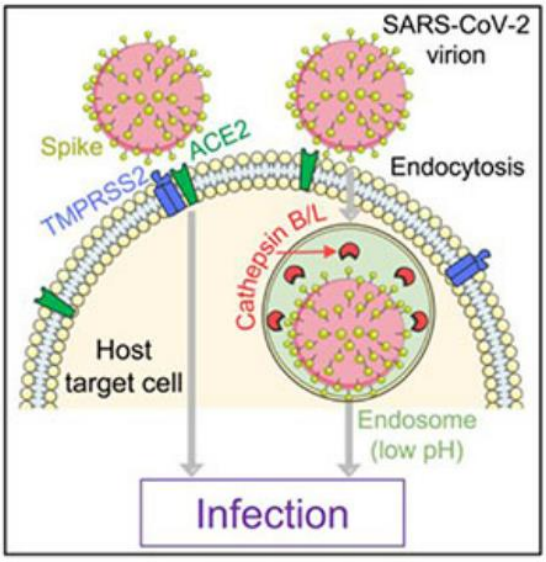

Figure 3. Schematic of the two independent entry pathways accessible to SARS-CoV-2 (35)

No symptoms are experienced during the initial phase of replication in the nasopharynx. This asymptomatic phase lasts 1-2 days and allows the virus to multiply in the upper respiratory tract without significant interference from innate immune cells. During this period, individuals are highly contagious, showing the highest viral load and infectivity at symptom onset (36, 37). Within 2-14 days after the initial encounter, the common symptoms of COVID-19 typically start to appear (15). In a systematic review and meta-analysis from J. Quesada *et al*., the mean incubation period, which is the time from infection to onset of symptoms, ranged from 5.6 (95% CI: 5.2–6.0) to 6.7 days (95% CI: 6.0–7.4)(38).

When the virus moves towards the lower respiratory tract via airways, a strong innate immune response is triggered. At this stage, patients exhibit an enhanced pro-inflammatory response. Increased number of cytokines including Interleukin-6 (IL-6), tumor necrosis factor α (TNF- α), macrophage inflammatory protein 1α (MIP-1α), granulocyte macrophage colony stimulating factor (GM-CSF) as well as chemokines (IP-10, CCL2/MCP1, CXCL1, CXCL5) are produced by the invaded cells. This leads to the attraction and accumulation of numerous immune cells like macrophages, neutrophils and dendritic cells in the lungs causing alveolar damage and edema and ultimately acute respiratory distress syndrome (ARDS) (15, 39, 40).

According to the ACE-2 distribution, SARS-CoV2 also invades other tissues, like the cardiovascular and gastrointestinal system where the cytokine storm leads to inflammatory changes, tissue degeneration, induction of a hypercoagulable state and necrosis. Additionally, the cytokine storm may induce viral sepsis, multiple organ failures, and death in the worst cases (15, 41).

1.1.4 Clinical presentation

COVID-19 encompasses a wide range of symptoms, spanning from asymptomatic infection to critical and fatal illness. Though, most affected individuals were either asymptomatic or had a mild clinical presentation (17.9 - 33.3%) (39).

As a respiratory disease, COVID-19 primarily affects the upper respiratory tract and causes symptoms such as fever, cough, shortness of breath in most symptomatic individuals. In addition, patients may experience sore throat, myalgia, malaise, and fatigue, diarrhea, and loss of smell and/or taste (39, 42). With the emergence of variant strains, including Delta and Omicron, mild upper respiratory symptoms like nasal congestion and sneezing have also become common (43). The primary severe symptom of COVID-19 is pneumonia, featuring a fever, cough, dyspnea, and bilateral pulmonary infiltrates (44).

COVID-19 can present in various forms, affecting not only the respiratory system but also impacting diverse bodily systems. Viral entry is facilitated through ACE-2 receptors, with the distribution of these receptors in various tissues influencing the virus's tissue-specific manifestations(32). Alongside the gustatory and olfactory abnormalities discussed previously, other neurological symptoms COVID-19 may present with are confusion, dizziness, and headaches. Gastrointestinal symptoms including nausea, vomiting, loss of appetite, abdominal pain, and diarrhea are prevalent. In addition, liver dysfunction may occur. Cardiac involvement can cause myocardial injury, myocarditis, acute myocardial infarction, heart failure, and arrhythmias. COVID-19 may additionally induce a hypercoagulable state, increasing the risk of thromboembolism in various areas, such as the lower limbs, pulmonary artery, and cerebrovascular system. This can result in subsequent complications. It is important to note these potential symptoms for early diagnosis and proper treatment. Lymphopenia is a frequent finding in laboratory settings. Observable characteristics may include thrombocytopenia, leukopenia, elevated ESR and CRP levels, high LDH levels, and leukocytosis (39, 45).

Acute respiratory distress syndrome (ARDS) is a significant complication observed in severe cases, which frequently arises shortly after the onset of dyspnea. It presents with rapid onset respiratory distress, severe gas exchange impairment, and widespread damage to the lungs' alveolar-capillary membrane. ARDS is clinically defined by the presence of hypoxemia and bilateral pulmonary infiltrates on chest imaging which cannot be fully accounted for by heart failure or fluid overload. In some cases, mechanical ventilation is required (46, 47).

Symptoms lasting longer than 8 weeks, 12 weeks after detected SARS-CoV-2 infection are classified as long- Covid/ post-Covid syndrome and still under research. A diverse range of symptoms has been reported, which may endure for weeks and months, reoccur in stages, or emerge anew. These encompass a wide array of manifestations including fatigue, exertional dyspnea, headache, anosmia and ageusia, rash and hair loss, concentration and memory problems, sleep disturbances, muscle weakness, and psychological problems such as depressive symptoms and anxiety. They may appear in previous healthy individuals, without pre-existing conditions (48, 49).

1.1.5 Severity

According to the National Institutes of Health (NIH) guidelines, COVID-19 is classified in 5 distinctive types.

Asymptomatic or pre-symptomatic Infection refers to individuals who test positive for SARS-CoV-2 but do not exhibit clinical symptoms consistent with COVID-19.

Mild illness encompasses individuals who display typical COVID-19 symptoms such as fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, anosmia, or dysgeusia. Importantly, they do not experience shortness of breath or show abnormal chest imaging.

Moderate illness is characterized by clinical symptoms or radiologic evidence of lower respiratory tract disease, and these individuals maintain an oxygen saturation (SpO2) level of 94% or higher when breathing room air.

Severe illness describes individuals with an SpO2 level below 94% on room air, a ratio of arterial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) of less than 300, marked tachypnea exceeding 30 breaths per minute, or lung infiltrates occupying more than 50% of the total lung volume.

Critical illness involves individuals experiencing acute respiratory failure with possible progression to ARDS, septic shock, or multiple organ dysfunction. (45).

The risk of a severe COVID-19 course increases with pre-existing conditions such as asthma, obesity with BMI >35, cardiovascular diseases, cigarette smoking, diabetes mellitus, chronic lung/liver/kidney diseases, organ transplantation and other forms of immunosuppression (for example untreated HIV infection) (39, 45).

1.1.6 Diagnostic methods

To prevent the spread of a viral communicable disease, like COVID 19, a rapid, non-invasive, low- cost, reliable and simple to use diagnostic is essential. Thereby, at an early stage, infection chains can be interrupted, and further measures conducted like treatment initiation for individuals at risk. Factors contributing to the overall usefulness of a diagnostic method are sensitivity and specificity, time expense for the test as well as results, costs, staff availability and infrastructure.

Diagnostic testing during the COVID-19 pandemic was suggested for individuals with COVID symptoms, high-risk exposures, screening purposes, or the termination of isolation. Notably, testing guidelines altered with progression of the epidemic and differed between regions and populations. Diagnostic testing has mainly involved the collection of samples from

the upper respiratory tract, with the nasopharynx being the main site. Other possibilities include the oropharynx, the nasal mid-turbinate or its anterior part, and saliva (50).

The diagnostic methods used in COVID-19 can be broadly classified into molecular testing and serological testing.

Molecular testing by a nucleic acid amplification test (NAAT), such as reverse transcriptase real-time polymerase chain reaction (RT-qPCR), is the preferred method for early detection, even before an individual develops symptoms. After a sample is taken, viral RNA is extracted, translated into DNA by reverse transcriptase (RT) and amplified in cycles (51, 52). Cyclic threshold amplification <35 was defined as RNA positive for most PCR types (53). RT-qPCR offers a highly specific and sensitive method and is therefore considered as the golden standard for COVID- 19 diagnosis. However, it should be noted, that false negatives may occur, if the amount of viral genome is insufficient, which applies to the incubation period. Therefore, false negative results within 7 days of infection are common. Additionally, RT- PCR result-turnover is time- consuming and requires test kits, which are expensive and commonly lead to shortage during epidemic outbreak (50, 54).

Rapid antigen tests based on the lateral flow principle are widely considered an alternative to expensive, labor-intensive PCR methods due to their simple and inexpensive performance. The pathogen antigens are qualitatively detected here by binding with, conjugated antibodies on test strips, which produces colored bands in the event of positive viral antigens. Antigen tests can be particularly useful in symptomatic individuals within the first five to seven days of symptoms when viral replication is at its highest. A positive antigen test in symptomatic individuals indicates SARS-CoV-2 infection, while a negative result should be followed by additional testing. In asymptomatic individuals following exposure, a negative antigen test should generally be followed by further testing. Due to simple usage, independent on laboratory, antigen testing can be employed for screening purposes, such as in outbreak settings and repeated screening of high-risk individuals. On one site, they enable rapid diagnostics on-site within 15 to 30 minutes. On the other site, rapid antigen tests have a lower overall sensitivity and are less reliable at low viral loads than PCR methods. In addition, most of these tests require a nasopharyngeal swab, which many individuals find uncomfortable (50, 54) .

Serological tests rely on the principle of antigen- antibody- specific binding by detection of produced antibody levels in the human blood serum. The main methods include the chemiluminescence immunoassay (CLIA), enzyme- linked immunosorbent assay (ELISA), lateral flow immunoassay (LFIA), and immunofluorescence assay (IFA) (24). Notably, depending on the type, antibody can be detected between 4 – 90 days for the first time after the

begin of COVID-19 infection (55). Therefore, these tests are not recommended for diagnosis of infection, but rather for assessment of past infections, immunity and population- level exposure to the virus (50, 54).

1.1.7 Prevention and vaccination

Preventive measures are crucial in controlling the spread of SARS-CoV-2. They serve not only to prevent the overload of the healthcare system and to regulate resources but also to protect individuals at risk of severe progression.

Personal preventive measures, such as hand washing, respiratory hygiene, and the use of hand sanitizer with at least 60% alcohol were recommended. Additionally, adequate indoor ventilation was advised. Especially, wearing of masks played a crucial role in preventing transmission and was often mandated in public places.

In addition to general COVID screening and testing, mandatory isolation protocols were implemented for individuals testing positive for COVID and their direct contacts. The duration of isolation and guidelines varied significantly among states and the stage of the pandemic but averaged around 14 days in most European countries.

Social distancing and national lockdowns were enforced by governments worldwide, limiting social activities and allowing citizens to leave their residences only for essential purposes such as grocery shopping. The effectiveness and sufficiency of these measures are still under retrospective evaluation (56).

As the COVID-19 pandemic progressed, vaccination became the most important strategy to control the virus. There are numerous types of COVID-19 vaccines, such as mRNA vaccines, vector-based vaccines, and protein subunit vaccines.

mRNA vaccines like Pfizer-BioNTech and Moderna vaccines, utilize a small segment of viral mRNA to instruct the host cells to produce the viral spike protein. This prompts a pre-adapted immune response, resulting in antibody and memory cell production.

Vector-based vaccines, including Johnson & Johnson and AstraZeneca vaccines, employ another virus, e.g., Adenovirus, as a vector to carry the genetic material of SARS- CoV2 to produce a modified spike protein. This activates an immune response, following a similar mechanism to mRNA vaccines.

In contrast, Novavax - an example of a protein subunit vaccine - directly delivers the spike protein, which is produced in vitro by recombinant technology before its injection. (39, 57).

The number of necessary vaccinations to achieve complete protection varies by vaccine, ranging from one to two injections. It is important to note that a vaccine's effectiveness differs depending on the viral strain and gradually declines over time (58). To combat waning immunity to COVID-19, boosters have been incorporated into the vaccination protocol. Overall, the World Health Organization (WHO) aims to achieve a minimum immunization coverage rate of 70% within the general population, with full coverage for high-risk groups and healthcare personnel (59).

1.1.8 Management and treatment

The clinical management of COVID-19 is a comprehensive approach that addresses symptom relief, support of the immune system, and the prevention of complications. To ensure the most effective treatment, healthcare providers assess the severity of the disease and any underlying conditions when a patient is admitted. Initial laboratory tests typically include a complete blood count (CBC) and a basic metabolic panel. For patients with severe COVID-19 or those requiring oxygen or ventilatory support, additional tests such as CRP, lactate dehydrogenase (LDH), prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen, D-dimer, troponin, and electrocardiogram (ECG) are conducted. Chest X-rays are used to evaluate pulmonary complications and exclude alternative diagnoses. They are instrumental in detecting parenchymal alterations like pleural effusion and irregular consolidations. In contrast, CT scans show multifaceted lung engagement marked by a distinctive "crazy-paving pattern." This pattern is defined by peripheral ground glass opacities and thickening of intralobular septa. (60-62).

Although COVID-19 treatment approaches have evolved over the course of the pandemic and vary between countries, they generally consist of both supportive and pharmacological measures. Supportive care primarily involves relieving symptoms through intravenous fluid therapy, antipyretics, analgesics, and antitussives. Additionally, oxygen therapy is employed to maintain oxygen saturation levels between 92% and 96%. Oxygen can be administered through nasal prongs, high-flow nasal cannula, or noninvasive ventilation. In more severe cases, oxygenation may require invasive ventilation, such as intubation, or in the most critical cases, extracorporeal membrane oxygenation (ECMO) (39).

The pharmacological treatment of COVID-19 encompasses a comprehensive strategy involving antiviral medications, monoclonal antibodies, corticosteroids, and immunomodulators. These treatment options are selected based on the severity of the disease and the specific phase of illness that a patient is experiencing.

During the early phase of COVID-19, characterized by heightened viral replication, antiviral drugs like Paxlovid (Nirmatrelvir/Lopinavir), Remdesivir, or Molnupiravir are utilized. These drugs function as adenosine nucleotide analogs, effectively inhibiting viral RNA replicase and replication. Their primary role is to target viral replication, making them particularly effective in the early stages of the disease.

Monoclonal antibodies, such as Sotrovimab or Bebtelovimab, are deployed to target the viral spike protein. However, their efficacy is influenced by the specific composition of the spike protein, making their success dependent on the circulating virus strains.

Corticosteroids, notably Dexamethasone, have demonstrated their effectiveness in reducing mortality rates, particularly in severe COVID-19 cases requiring oxygen supplementation or ventilatory support. However, mild cases not necessitating supplementary oxygen do not derive substantial benefit from corticosteroid treatment. In moderate to severe cases requiring ventilatory support, Dexamethasone is commonly combined with Remdesivir.

Immunomodulators like Tocilizumab, an anti-IL-6 monoclonal antibody, target the IL-6 receptor to reduce inflammation. Janus kinase (JAK) inhibitors such as Baricitinib or Tofacitinib reduce inflammation and inhibit cytokine signaling and activity.

To address the hypercoagulable state and the increased risk of venous thromboembolism, prophylactic heparin is recommended. However, therapeutic anticoagulation is not advised for critically ill patients (63, 64).

1.1.9 Virus variants of SARS-CoV2

Coronaviruses, like SARS-CoV2, are characterized by high mutations rates in RNA replication, genome modification as well as recombination (65). This results in the emergence of new virus strains, allowing the virus to rapidly adapt to its host, which is crucial for its survival. Moreover, virus evolution is propelled by selection pressure from the environment, increasing host immunity, and antiviral treatments (66). In this regard, mutations in the RBD of the S protein appear to have the most significant impact. The spike protein's binding to the ACE receptor is not only a crucial step for viral cell entry, cell range, and tissue tropism but also makes it a key antigen within the viral membrane when interacting with the host. Within the host, this interaction stimulates the production of a substantial number of neutralizing antibodies. (65) However, even single substitutions or mutations in the receptor-binding domain (RBD) can lead to immune evasion, causing the neutralizing antibodies to lose their ability to recognize the virus. While these mutations can enable immune evasion, they can also have

detrimental effects, including increased transmission, heightened virulence, greater disease severity, and reduced effectiveness of therapeutic interventions and vaccines.

During the COVID 19 pandemic, different virus variants evolved. The WHO defined variants of interest (VOI) and variants of concern (VOC). VOIs have scientifically proven genetic changes in virus characteristics and growth advantages. If they meet additional criteria such as changes in disease severity, impact on the healthcare system, or reduced vaccine effectiveness, they are classified as VOCs.

The Alpha variant (B.1.1.7 lineage) of SARS- CoV2 was initially recognized in the United Kingdom in September 2020. It is known for its huge number of mutations (N:23), from which 8 are located at the spike protein. The mutation N502Y within the receptor-binding domain (RBD), for example, is characterized by increasing binding affinity to the human ACE 2- receptor, which results in increased infectiousness, as less virus load for infection is needed. Another mutation, E484K, the so-called escape mutation influences immune response and efficacy of vaccinations. Furthermore, immune evasion in immunocompromised patients and increased transmission was detectable. Overall, the alpha variant showed an increased mortality compared to earlier variants.

Within the same time as alpha was identified, a new variant was found as well in South Africa, named B. 1.351 lineage. It contains 9 mutations, from which 3 (K417N, E484K, N501Y) are in the RBD. Like the alpha variant, beta shows increased transmission. Further mutations lead to immune evasion, higher contagiousness, decreased efficacy of monoclonal antibody therapy and decreased neutralization by antibodies.

The SARS- CoV-2 variant P.1 (Gamma) was first detected in the Brazilian Amazonas state. Like the other variants, it shows several spike protein polymorphisms and resembles the south African variant. Within 17 amino acid changes, 10 are included in the S protein, 3 of them of critical concern: N501Y, E484K, K417T. The Gamma variant is also associated with increased transmissibility, reduced effectiveness of neutralizing antibodies, and increased disease severity.

Discovered in India, in May 2021, the WHO declared lineage B. 1.617.2 (Delta) as VOC. It is associated with 40- 60% increased transmission compared to alpha, higher case fatality rate, severity of diseases as well as hospitalization rates. In addition, vaccination efficacy is highly reduced. Delta was the dominant variant in most countries around the world in the second half of 2021.

Omicron was characterized as VOC on November 26, 2021. Compared to the SARS-CoV- 2 strain in Wuhan, B.1.1.529 possesses the high number of 30 amino acid changes within

the spike- protein. Some of the mutations are known to increase transmission and immune evasion, resulting in 2,8 increased contagiousness compared to Delta. Additionally, increased risk of reinfection, compared to other variants was found. Though, Omicron showed decreased virulence compared to the wild type. Since February 2022, it is the prevailing type in Germany (Figure 3) (64, 67).

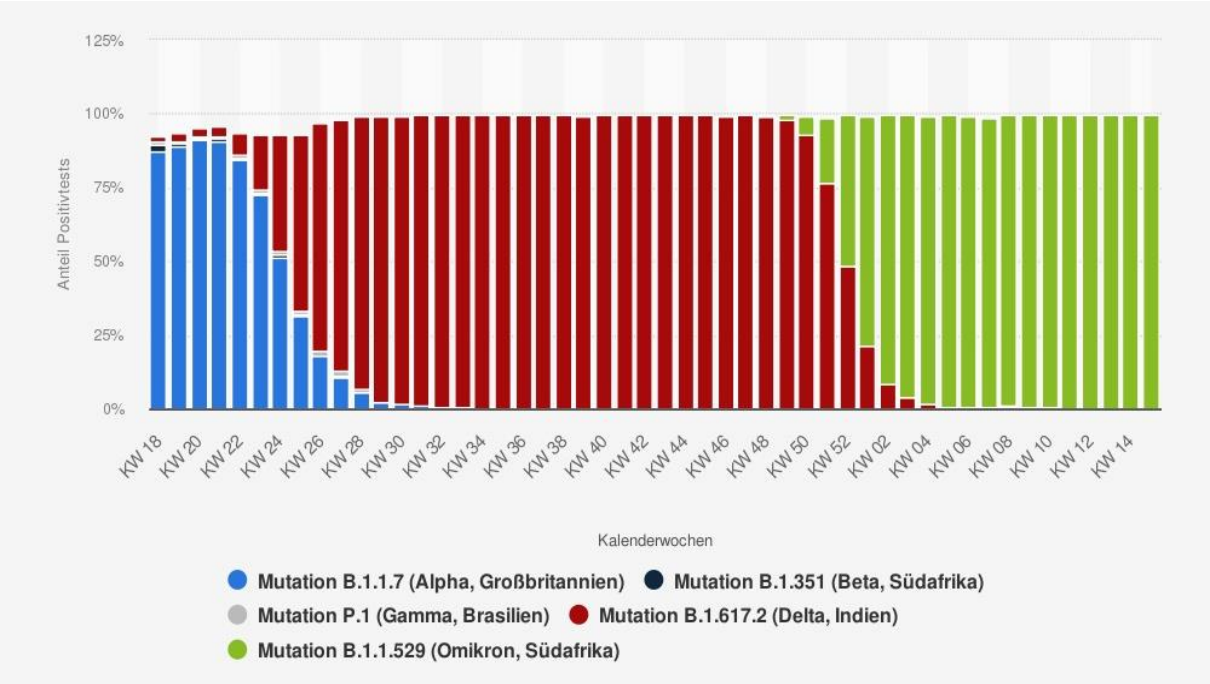

Figure 3. Development of the SARS CoV-2 VOC variants in 2021/2022, x axis - calendar week of 2021/2022, y axis Proportion of SARS CoV-2 detected variants (68)

1.2 Volatile organic compounds

1.2.1 Scent

In ancient times, physicians diagnosed diseases through various senses, including hearing, seeing, and smelling. Today, medical diagnosis relies mainly on seeing and interpreting the results of invasive technological devices. In the past, diabetes was identified by the sweet breath caused by acetone, and liver disease by a fishy odor. However, even today, certain scents are considered in medical school to be specific to certain diseases or pathogens. Diarrhea caused by the bacterium Clostridium difficile produces a foul odor, while Pseudomonas (P.) aeruginosa in culture has a grapelike scent, caused by 2` aminoacetophenone (69, 70).

1.2.2 VOCs as biomarker of disease

Volatile Organic Compounds (VOCs) are responsible for the characteristic, noticeable odors mentioned above. Volatile organic compounds encompass a broad range of chemical compounds with low molecular weights and high vapor pressures. Examples of VOCs include terpenes, aldehydes, alkanes, alkenes, esters, and aromatics. These compounds arise from diverse sources such as natural processes, industrial activities, and biological functions in living organisms. The human body, for instance produces various odorous and non-odorous VOCs that can be detected in exhaled breath, urine, sweat, blood, and other bodily fluids. The VOCs emitted from diverse areas of the body differ based on age, diet, sex, physiological status, and potentially genetic background, and thus can be considered as individual ‘odor-fingerprints’ (71, 72).

VOCs have the potential to function as biomarkers, which are measurable indicators of normal or abnormal biological processes, pathogenic conditions, or responses to treatment. Specific VOCs are produced in altered metabolic pathways and changes in gut microbiota. In addition to that, oxidative stress and inflammation leads to alterations in VOC production. Therefore, specific VOCs have been linked to certain diseases like cancer, diabetes, respiratory diseases, and gastrointestinal disorders (73-78). These VOC profiles associated with particular diseases have the potential to be used for early diagnosis and monitoring, leading to timely intervention and better patient outcomes.

1.2.3 VOC analysis in breath

Exhaled breath contains a variety of VOCs that can be attributed to either exogenous or endogenous volatiles. Exogenous volatiles comprise compounds inhaled from the external environment, compounds produced following oral ingestion of food, and those derived from smoking cigarettes. Endogenous volatiles consist of compounds carried by the blood that are released into the environment through the lungs, as well as those produced by all types of symbiotic bacteria (71). Notably, bacteria grow next to cells and produce their own VOCs, whereas viral pathogens replicate inside cells without producing their own VOCs but altering the metabolic pathway of their host, such as inducing aerobic glycolysis or fatty acid synthesis (79).

Since the concentrations of VOCs in breath are detected at nanomolar to picomolar levels, differentiating endogenously produced VOCs from contaminant environmental exogenous compounds can be challenging. Nonetheless, collecting breath samples is straightforward, painless, and non-invasive. Therefore, numerous analyses of breath samples have been conducted, and in certain instances, scientists and medical professionals have successfully detected VOCs that are unique to diseases. For instance, trimethylamine has been detected in the breath of individuals with trimethylaminuria and methyl mercaptan in the breath of patients with fetor hepaticus (71).

The composition of VOCs in breath is affected by the sampling method and the depth of breathing. On average, 150 ml of breath is expelled from the upper airways (nose, throat, and trachea) during normal breathing. The remaining air is predominantly from the alveolar region, which can be accessed via deep exhalation, allowing metabolic VOCs to be extracted from the body and transported to the lungs via the bloodstream. Additionally, the breath can be gathered through the mouth or nose, depending on the circumstances. In the case of infections of the nasopharynx (such as influenza), sampling nasally may be more appropriate, while deep exhalation may be more suitable for sampling lung cancer.

Breath analysis is an attractive option due to its ability to provide point-of-care location, rapid results (< 10 min) without relying on reagents, non-invasive sampling with low biosecurity burden, and applicability in a wide range of scenarios worldwide, including low-resourced environments like community or primary care settings (80).

1.3 Gas-chromatography coupled ion mobility spectrometry (GC-IMS)

Gas Chromatography-Ion Mobility Spectrometry (GC-IMS) is a hybrid analytical technique that combines the high selectivity of gas chromatography (GC) with the extraordinary sensitivity of ion mobility spectrometry (IMS) to separate and identify complex mixtures (81).

1.3.1 Gas Chromatography (GC)

GC is a chromatographic technique utilized for the separation and analysis of individual compounds from a complex mixture, like volatile and semi volatile compounds in gaseous or vaporized form. Separation occurs based on chemical properties, such as volatility and polarity. In GC, a sample is volatilized and introduced into a chromatographic column, where it is propelled through a stationary phase by a carrier gas. The separation process occurs due to differential interactions between the sample components and the stationary phase. As compounds traverse the column at distinct rates, they elute sequentially and are detected by a suitable detector, thereby generating a chromatogram that portrays compound abundance as a function of time (82).

1.3.2 Ion Mobility Spectrometry (IMS)

After separation in the GC column, the individual compounds are directed into the IMS portion of the instrument. IMS is a technique that measures the mobility of ions in a drift tube under the influence of an electric field. The mobility of ions is influenced by their size, shape, and charge. In IMS, the separated compounds are ionized, typically by using ionization sources like corona discharge or radioactive ionization, and then passed through an electric field. The ions move through the drift tube against a carrier gas at different rates based on their properties, creating distinct arrival time distributions (83).

1.3.3 Detection and Analysis

The ions' arrival time distributions are detected and recorded by the IMS detector. Each compound in the sample produces a unique mobility spectrum, often referred to as an ion mobility fingerprint, which is highly characteristic of the compound's structure and aids in identification. The ion mobility data are typically presented as a 2D plot, with retention time from the GC on one axis and ion mobility expressed as drift time on the other.

1.3.4 Identification

Identification is based on the unique combination of retention time and ion mobility characteristics for each compound. The retention time in GC part and the drift time in the IMS part, together, describe each unique molecule. The resulting data can be compared to databases of known compounds to identify the components in the sample.

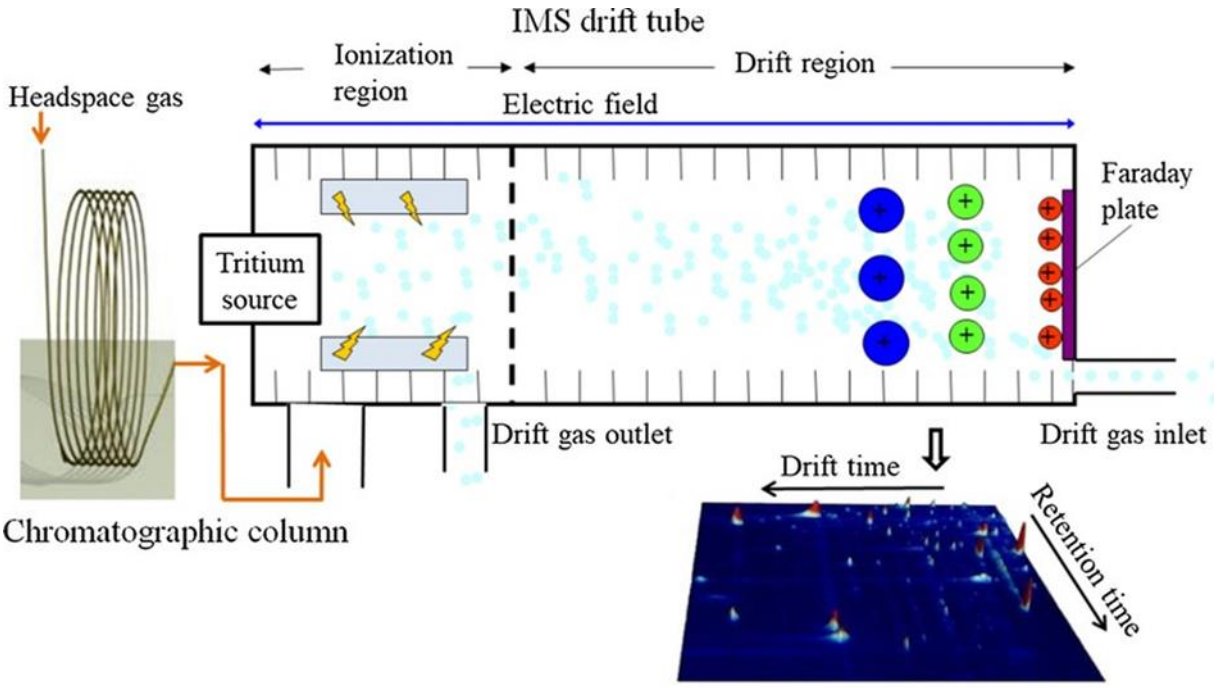

Figure 4: Schematic of GC-IMS (84)

GC-IMS has several advantages, including high sensitivity, fast analysis times (<5min), and the ability to analyze complex mixtures of volatile compounds (81) Furthermore, a pre-treatment of the sample is not required in GC-IMS , the devices have good portability, are relatively cheap, easy to use, and deliver reproducible results (73). Therefore GC- IMS is used in various fields, including environmental monitoring to detect pollutants and VOCs, food and beverage analysis to identify flavor compounds and contaminants as well as security and defense for the detection of explosives and illicit substances (85-87). In recent years, due to its ability to rapidly identify and quantify volatile compounds, it also gained interest in medical diagnostics to analyze breath or other biologic samples on disease markers (80, 88).

2 OBJECTIVES

2.1 Aims of the study

Rapid, noninvasive, and practical diagnostic of viral diseases in epidemics or pandemics is essential to interrupt infection chains at an early stage. A promising approach for non-invasive diagnostics is the analysis of volatile organic compounds (VOCs) as biomarkers found in exhaled breath. In this study, the feasibility of testing COVID-19 positive patients by GC-IMS through exhaled breath was investigated. GC- IMS, based on the detection of volatile organic compounds, offers new possibilities that could provide new screening methods.

2.2 Hypothesis

COVID 19 positive patients can be detected by GC-MS through exhaled breath.

3 SUBJECTS, MATERIALS AND METHODS

3.1 Ethical Approval

The study was approved by the ethics committee of the university of Erlangen (Report No 426_18B). It was conducted in accordance with the WHO principles of the Declaration of Helsinki, Good clinical practice, and the European General data protection regulation. All patients gave written informed consent prior to participation.

3.2 Study Design

An experimental, non-randomized controlled study was performed to assess the testability of SARS-CoV2 via exhaled breath using GC-IMS. Patients hospitalized at REGIOMED hospital in Coburg between April and July 2022 were PCR tested for SARS-CoV2 via nasopharyngeal swab, following hospital COVID-19 pandemic policies. Within 24 hours eligible patients (> 18 years) were then asked to participate in the study. Exhaled breath was subsequently collected and analyzed using GC-IMS.

3.3 Participants

Patients were classified as either positive or negative for SARS-CoV2 based on their PCR test results. No exclusion criteria were defined for SARS-CoV2 positive patients, except for age over 18 and conscious content providing and participation. For SARS-CoV2 negative patients, we ensured that there had been no prior COVID-19 infection within the past three months due to potential changes in the VOC composition from pathophysiological alterations. Out of the 99 patients recruited data from 90 individuals were analyzed for the results. 9 patients were excluded, as they were not meeting the inclusion criteria or insufficient and missing data on the GC-IMS were recorded.

3.4 PCR

SARS-CoV-2 was tested by obtaining a deep nasopharyngeal swab using the `Xpert Nasopharyngeal Sample Collection Kit for Viruses (Cepheid, Maurens- Scopont, France). RNA was extracted from the sample using the StarMag 96 UniTube Kit (Seegene) on the SGPRep32 extraction system (Seegene), after which real-time PCR was conducted using the Allplex 2019-nCoV Assay (Seegene, Seoul, South Korea) on the cfX 96 Real-Time System (BioRad, Feldkirchen, Germany) targeting the E, N, and RdRp genes.

3.5 Breath sampling and GC-IMS

Patients' breath was analyzed with the GC-IMS during the first 24 hours after PCR testing to ensure sufficient viral load in the upper respiratory tract. After each measurement, the sampling system was changed.

The GC- IMS device from STEP Sensortechnik and Elektronik, Pockau, Germany (STEP IMS NOO) was used. The device is distributed as medical device (In- vitro- diagnostic) in combination with evaluation software as the MultiMarkerMonitor by Graupner medica solution GmbH, Geyer, Germany.

All patients received a single- use PULMOSAFE 3 viral & bacterial filter with an oval mouthpiece (Lemon medical GmbH, Hammelburg, Germany) which was connected by a polyethylene pipe to the GC- IMS. They were then instructed to inhale deeply and exhale slowly through the oval mouthpiece. Breath was sampled for 10s.

The STEP device operates without the need for any pre-analytical procedures. It uses an internal pump capable of delivering a flow rate of 200 ml per minute to draw the sample directly into the analysis circuit. The pump efficiently fills a loop made from perfluoro alkoxy polymer, with the flow maintained at 200 ml per minute. The filling time of this loop can be precisely controlled in fractions of seconds. It must be ensured that the loop is filled with the sample. During the filling process the sample is drawn trough the loop and leaves the device by the waste gate. It is ensured that the loop is filled with 2 ml of the sample after stopping the pump. These 2 ml are let into the GC by a valve. The GC (60°C) then pre- separates the sample into individual analytes according to their retention times. In the IMS unit the analytes are ionized by beta radiation of tritium source below the free limit for radiation (99MBq). Afterward, the generated ions are accelerated in a 50mm long drift- tube under the influence of an electric field (400V cm^{-1}) toward the detector, which is tempered to 60°C. On their way, the positive ions collide with air molecules from the drift gas flowing in the opposite direction and are separated according to their ion mobility and detected by the collector electrode, which is sampled every 10 micros. The ion gate has an adjustable opening time, currently set at 100 microseconds, and is pulsed every 30 milliseconds. The device records 16 single spectra per second, which are then averaged and undergo a wavelet transformation. The resulting denoised spectrum is used for further analysis. The received IMS spectra are stored internally and analyzed offline later. The IMS device utilized is equipped with an internal gas circulation and a circulation filter. The device was supplied with ambient air using a circulation pump, with activated carbon filtered drift (400ml min-1) and carrier (20 ml min-1) gas. The STEP device uses filtered air and does not need an external gas supply.

3.6 Data analysis

The volatile organic compounds (VOCs) are identified by their retention time in the GC and drift time in the IMS. Every 10qs (totaling 20.48ms), one spectrum over 2048 measurement points is obtained every second for a total duration of 240s. These spectra can be displayed on a heatmap with the retention time on the y-axis and the drift time on the x-axis. To simplify the data, we employed a proprietary cluster analysis software that uses a support vector machine. After baseline correction for noise, the software identifies the peaks of each measurement using the intensity signal threshold and categorizes them according to retention time and drift time. Based on these parameters, the clusters are numbered with the assumption that each cluster represents a distinct VOC. Peaks from different measurements, with similar drift and retention times according to a defined threshold, are mapped to the same clusters.

3.7 Variables

The collected independent variables comprise age, gender, ORBIS case number and the study participant number. ORBIS is the hospital`s data collection system which was used for PCR CT value assessment. Exhaled breath volatile composition was the dependent variable in this study. Confounding variables included temperature on the day of the GC-IMS test, vaccination status, symptoms (cough, fatigue, headache, myalgia...) and previous COVID-19 infections.

3.8 Statistical analysis

For statistical analysis IBM SPSS 25 (IBM, Armon, NY) was used. To assess and compare the samples regarding their variables, descriptive statistics in the form of mean, median, standard deviation, minimum and maximum were used. For comparison and assessment of the significance regarding the distribution of the independent variables, as no normal distribution could be assumed, data were analyzed using the Mann-Whitney U test. To test for the presence of an association or relationship between two categorical variables, the chi-squared test was used. The significance level was set at a p value of <0.05. Furthermore, to analyze the association between two categorical variables, in four-field tables, the study used the Fisher's exact test.

To excluded cross- correlated clusters, received by GC-IMS, we performed a stepwise canonical discriminant analysis to optimally minimize Wilks lambda. Significances of 0.05 and 0.1 were used to enter or remove variables from the model.

4 RESULTS

4.1 Demographic analysis and variables

A total of 89 patients (>18 years) who were hospitalized in the REGIOMED hospital Coburg between April and July 2022 were enrolled, including 4 staff members. 48 of them ware male (53,9%) and 41 females (46,1%) (Table 1). Among males, the mean age revealed was 63, 23 with a standard deviation of 16,7, whereas females were on average 62.1 years old (SD: 22,9).

The mean age of all patients was 62,7 (SD: 19,7). A rank-sum (Mann-Whitney) test was conducted to assess any significant differences in age between genders. The test revealed a non-significant result ($z = 0{,}479$, $p > 0.05$), suggesting that there were no significant differences in age between males and females in the study population.

As illustrated in table 1, 58 of the 89 patients recruited, tested positive for SARS-CoV-2 by RT-qPCR. Among positives, there were 35 males and 23 females. Negative tested were 13 males and 18 females (Table 1). The mean age of positive tested patients showed to be 64 (SD: 18,5) while negatives were 60,4 years old (SD: 21,8). Independent- Samples Mann-Whitney U test was conducted to investigate the distribution of age across positives. No significant relationship between age and positivity was found ($z = 0{,}567$, $p<0{,}05$).

Table 1. Study population

			RT-q PCR for SARS CoV-2		
		Group	**negativ**	**positive**	**Total**
Gender	male	Count	13	35	48
		% within gender	27,1	72,9	100,0
	female	Count	18	23	41
		% within gender	43,9	56,1	100,0
Total		Count	31	58	89
		%	34,8	65,2	100,0

Fisher's exact test assessed the correlation between genders in the RT-qPCR SARS CoV-2 positive-tested study population. Our findings did not reveal any significant difference between genders among positives; the p-value of 0.12 was greater than 0.05.

RT-qPCR SARS CoV-2 positive tested individuals showed to have a mean CT value of 25,93 (SD: 5,70). By conducting the Independent- Samples Mann- Whitney U Test ($z= 0,81$, $p<0,05$), no significant correlation in regard of distribution of CT values among genders was found.

4.2 Cluster analysis

Table 2: Cluster analysis

Cluster	rt (s)	dt (ms)	Cluster	rt (s)	dt (ms)
C_1	29,02	39,9	C_27	133,36	43,15
C_2	15,86	7,57	C_28	136,42	9,37
C_3	22,05	14,17	C_29	136,67	4,46
C_4	21,91	20,72	C_30	161,08	7,83
C_5	28,21	3,24	C_31	165,93	11,37
C_6	25,21	28,45	C_32	161,37	43,67
C_7	41,24	22,88	C_33	161,86	2,44
C_8	34,13	34,36	C_34	31,52	49,71
C_9	33,99	64,31	C_35	112,43	17,05
C_10	48,7	44,1	C_36	104,46	78,04
C_11	45,3	8,84	C_37	120,38	35,11
C_12	51,02	28,9	C_38	173	21,15
C_13	50,94	16,94	C_39	40,33	56,78
C_14	56,01	51,97	C_40	68,24	10,88
C_15	84,72	43,1	C_41	82,19	30,44
C_16	68,63	5,09	C_42	140,44	13,74
C_17	86,45	8,96	C_43	36,22	67,82
C_18	89,45	20,04	C_44	72,87	47,41
C_19	99,97	13,74	C_45	103,33	1,69
C_20	87,53	24,62	C_46	111,17	45,07
C_21	102,5	97,5	C_47	160,47	48,09
C_22	107,61	41,64	C_48	122	54
C_23	116,16	7,84	C_49	36,75	74,88
C_24	117,26	21,85	C_50	91,65	81,22
C_25	134,57	19,84	C_51	45	87
C_26	126,81	29,58			

Clusters are categorized by retention time (rt) and drift time (dt) according to their position in the IMS chromatogram.

51 clusters were found after analysis in the GC- IMS, as can be seen in table 2. They showed retention times in the range of 15,86- 161,86ms and drift times in the range of 3,24-97,5ms. In a stepwise approach, clusters were further analyzed in the canonical discriminant analysis. The relevant clusters revealed were C_18 (ts :89,45ms, dt: 20,04ms), C_20 (ts:84,53ms dt: 24,62ms) and C_30 (ts:161,08ms, dt: 7,83ms) (Figure 5).

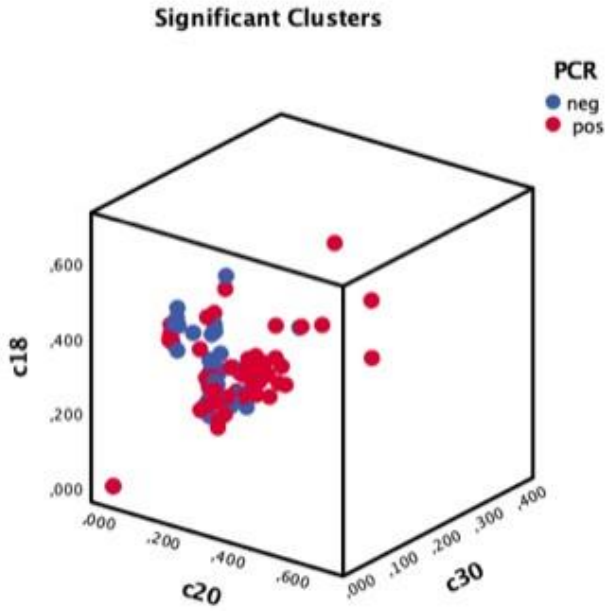

Figure 5: Significant clusters visualized in a 3D model.

Table 3: Classification results

			Predicted Group Membership		
positive		**Group**	**Control**	**SARS CoV-2**	**Total**
Original	Count	Control	23	9	32
		SARS CoV-2	12	45	57
	%	Control	71,9	28,1	100,0
		SARS CoV-2	21,1	78,9	100,0
Cross validated	Count	Control	22	10	32
		SARS CoV-2	12	45	57
	%	Control	68,8	31,3	100,0
		SARS CoV-2	21,1	78,9	100,0

76,4% of original grouped cases correctly classified.
75,3% of cross- validated grouped cases correctly classified

Table 3 shows the classification results of cluster analysis. 45 out of 57 tested COVID-19 patients were correctly classified as positive, while 23 out of 32 were correctly classified as negative. A sensitivity of 78,9% and a specificity of 71,9% can be calculated thereby. Overall, 76,4% of original grouped cases were correctly classified in the original classification. For verification, a cross- validation was conducted which showed 75,3% of correct classification of groups.

5 DISCUSSION

RT-qPCR is the current gold standard for COVID-19 diagnosis. Despite its high sensitivity and specificity, its expense and special kit requirements limit its use to developed countries. Additionally, the turnover time of at least 30 minutes for results is not feasible for places requiring widespread population screening within a short period of time, such as at airports. Antigen tests offer a cost-effective PCR alternative. Although test results are available within minutes, they lack high sensitivity (overall 70%) (89). Additionally, PCR and antigen tests require an invasive nasopharyngeal swab, which especially children and elderly find inconvenient (90). It is important to note that nasopharyngeal swabs are effective only during the initial viral replication phase in the nasopharynx. Once the virus migrates towards the lower lung compartments, the swab no longer picks up the virus. In contrast, breath analysis offers the potential to assess all regions of the respiratory system as it is measured by end tidal volume deep in the lungs, and therefore presents a promising avenue for improved diagnostics.

In this study, the exhaled breath of COVID-19 positive patients was collected and analyzed using GC-IMS to determine the feasibility of this method. GC-IMS detects volatile organic compounds and offers a quick, non-invasive, and practical diagnostic method potentially for point-of-care screening purposes.

Among the study population of 89 patients, 58 tested positive for SARS-CoV-2 using RT-PCR with a mean Ct value of 25.9. No significant differences were found between the groups in terms of age and gender. The GC-IMS analysis of breath revealed 51 Clusters, of which 3 appeared to be relevant for COVID-19. It was shown that 76.4% of the original grouped cases were correctly classified, while 75.3% of cross-validated grouped cases were correctly classified. Sensitivity appeared to be 78.9% and specificity 71.9%.

With a similar study design *Ruszkiewicz et al*. investigated exhaled breath testability with GC-IMS in 2020 (80). Breath sampling was done separately and subsequently manually injected into the GC-IMS with a syringe, which requires trained personnel. Additionally, breath was not sampled within a specific period but rather at any time during hospital stay. As the study comprised the summary of two independent smaller studies in Edinburgh and Dortmund, the results showed 80% and 81.5% accuracy, sensitivity of 82.4%/90% and specificity of 75%/80%. While *Ruszkiewicz et al.* included various pre-existing conditions known to lead to different VOC profiles in the results, they were not taken into account in the present study and pose potential bias. However, it should be noted that a suitable screening method ideally distinguishes SARS-CoV2 or other viral diseases without knowledge of pre-existing conditions. Furthermore, specific VOCs for COVID-19 were found in the study. Nonetheless, metabolic origin of these substances could not be determined and does not seem to be detrimental for

differentiation of SARS- CoV2. This assumption is emphasized by the fact, that dogs are able to sniff SARS-CoV2 as well as various forms of cancer with high sensitivity and specificity, without prior knowledge of VOC composition (91, 92).

SARS-CoV-2 detection in exhaled breath was also assessed by multicapillary column coupled ion mobility spectrometry (MCC-IMS) (93). Unlike GC-IMS which uses a single long chromatographic column, MCC-IMS employs multiple capillary columns for pre-separation. Though slower, it yields higher resolution. *Steppert et al*. tested for not only SARS-CoV-2 but also Influenza in exhaled breath. 72 out of 74 were correctly classified as either SARS-CoV2 or Influenza and could be differentiated from each other without determining the exact VOCs. While the study measured exhaled breath within a one-month time frame, our measurement duration extended for four months, encompassing additional confounding factors that could potentially affect VOC production and composition.

The electronic nose offers an alternative approach for SARS-CoV2 testing (94). It employs several gas sensors that are combined with a pattern recognition system to analyze and characterize sample-derived complex VOCs without separation of the mixture into individual components. Nonetheless, gas sensors are predetermined to specific substances and not disease specific. Additionally, the gas sensors are sensitive to variations in atmospheric conditions (e.g., humidity, temperature), thus leading to varying sensing results depending on the initial placement of the electronic nose. In the study by *Nurputra et al*. the system demonstrated detection accuracy of 88–95%, sensitivity of 86–94%, and specificity 88–95%. However, patients were required to fast for 1 hour prior to testing. Furthermore, breath sample was collected in a separate bag that subsequently had to be connected to the electronic nose manually for further analysis. This not only results in sample leakage and dilution with ambient air during reconnection of the sampling system but is also impractical for rapid population screening.

While the beforementioned studies were conducted before 2022 where Alpha, Gamma and Delta were the prevailing SARS- CoV2 variants, in our study frame Omicron was the dominant variant. Omicron, despite its increased contagiousness, showed decreased disease severity. The abundant number of spike protein modifications of Omicron thus potentially affect the host cell metabolism in a reduced way compared to earlier variants and therefore also the VOC production. GC- IMS cluster recognition and differentiation is dependent on VOC alterations. Accordingly, the reduced VOC production potentially explains the lowered accuracy received in our study.

Considering the mean Ct value of 25.9, this indicates a low overall viral load in all patients, as Ct values are inversely correlated with the viral load in a specimen. According to a meta-analysis and systematic review by *Khalid et al*., the sensitivity of rapid antigen tests for SARS-CoV2 was shown to be 96% (95% CI: 95–97) among patients with a high viral load (Ct value ≤25). However, the sensitivity of rapid antigen tests dropped to 69% in patients with a low viral load (Ct value >25) (89). Another systematic review and meta-analysis revealed a decline in the sensitivity of rapid antigen tests to 10.8% in patients with Ct values greater than 25 in Omicron variant (95). This finding could also account for the moderate accuracy and sensitivity in our results, as low viral load leads to decreased cell invasion and therefore diminished VOC production.

Although our hypothesis has been confirmed that COVID-19 can be detected in exhaled breath through GC-IMS, the modest accuracy obtained raises concerns regarding the suitability of GC-IMS as a screening method. A screening method is defined by, among other things, having high sensitivity and specificity, ideally reaching 100%. While this level is rarely achieved, ensuring high sensitivity is crucial in accurately identifying individuals with a disease, particularly those who are asymptomatic. The initial step, therefore, is to test with high sensitivity. In a subsequent stage, individuals can be accurately ruled out if they test negative for the disease with high specificity. Due to slightly higher sensitivity compared to specificity, our findings suggest that GC-IMS could serve as a pre-screening tool before secondary confirmatory molecular tests. Nonetheless, more research is necessary to evaluate the exact accuracy of GC- IMS.

There are certain limitations to consider in this study. Firstly, several factors such as medication, nutrition, smoking, pre-existing medical conditions, and the composition of the surrounding air influence the VOC composition and ultimately the peaks of identification. However, due to the study's simplicity, these factors were not considered. Secondly, the study has limitations due to its small sample size, not only in terms of the number of participants but also the ethnicities represented, which could potentially alter VOC composition via genetically modified metabolism. Additionally, in a direct comparison between individuals with a viral infection and those without, the identified VOCs may also be a result of the host's response to a non-specific viral infection. Moreover, this study lacked reference database as well as training data sets and data analysis algorithms.

6 CONCLUSION

To conclude, the results of this experimental case control study demonstrate that COVID-19, as an example of viral communicable disease, can be detected by GC- IMS through exhaled breath. The measurement of exhaled breath and its analysis by GC- IMS based on VOC composition is non- invasive, fast (<5 min), easily performed and readily available test, and offers a promising tool for a point- of care screening or pre- screening method. However, due to moderate accuracy in this study, further broad- population, experimental studies are required to validate the diagnostic accuracy of exhaled breath analysis by GC-IMS.

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8 SUMMARY

Background: The COVID-19 pandemic with its worldwide spread, plays an example of viral communicable disease. A rapid, non-invasive diagnostic method is essential to interrupt early infection chains. RT-PCR tests are the current golden standard for COVID- 19 diagnostic, though infeasible for broad population screening due to expense, special kit requirements and prolonged result turnover. Volatile organic compounds analyzed in exhaled breath by GC- IMS offer a promising quick, non- invasive, and practical screening tool.

Methods: An experimental, non-randomized controlled study was performed to assess the testability of SARS-CoV2 via exhaled breath using GC-IMS. Patients hospitalized at REGIOMED hospital in Coburg between April and July 2022 were PCR tested for SARS-CoV2 via nasopharyngeal swab, following hospital COVID-19 pandemic policies. Within 24 hours eligible patients (> 18 years) were asked to participate in the study. Exhaled breath was subsequently collected and analyzed using GC-IMS. The collected data were statistically evaluated using IBM SPSS 25. Descriptive statistics, the Mann-Whitney U test, the chi-squared test and Fisher's exact test were applied with p- value set at <0.05. To identify significant VOCs in GC-IMS spectra, software based on cluster analysis followed by multivariate statistical analysis were employed.

Results: 89 patients were measured and analyzed in this study. 58 individuals tested positive for SARS- CoV-2 with RT-PCR and a mean CT value of 25.9. No significant differences were found between the groups in terms of age and gender. The GC-IMS analysis of breath revealed 51 Clusters, of which 3 appeared to be relevant for COVID-19. The classification results showed that 76.4% of the original grouped cases were correctly classified, while 75.3% of cross-validated grouped cases were correctly classified. Sensitivity appeared to be 78.9% and specificity 71.9%.

Conclusion: The results of this experimental case control study demonstrate that COVID-19, as an example of viral communicable disease, can be detected by GC- IMS through exhaled breath. The measurement of exhaled breath and its analysis by GC- IMS based on VOC composition is non- invasive, fast (<5 min), low- cost, easily performed and readily available test, and offers a promising tool for a point- of care screening or pre-screening method.

However, due to the moderate accuracy in this study, further broad-population, experimental studies are required to validate the diagnostic accuracy of exhaled breath analysis by GC-IMS.

9 CROATIAN SUMMARY

Naslov: Otkrivanje infekcije COVID-19 u izdahnutom zraku pomoću plinske kromatografije spojene s ionskom pokretljivošću (GC-IMS)

Ciljevi: Pandemija COVID-19 s globalnim širenjem predstavlja primjer zarazne bolesti uzrokovane virusom. Brza, neinvazivna dijagnostička metoda ključna je za prekid rane lanca infekcija. RT-PCR testovi trenutno predstavljaju zlatni standard za dijagnostiku COVID-19, iako su neizvedivi za široko testiranje populacije zbog visokih troškova, specifičnih zahtjeva za opremom i dugotrajnih rezultata. Analiza hlapljivih organskih spojeva u izdahnutom zraku putem GC-IMS nudi obećavajuće brzo, neinvazivno i praktično sredstvo za probir.

Materijali i metode: Izvedena je eksperimentalna, ne-randomizirana kontrolirana studija kako bi se procijenila testirajuća sposobnost SARS-CoV2 putem izdisanog zraka koristeći GC-IMS. Pacijenti hospitalizirani u bolnici REGIOMED u Coburgu između travnja i srpnja 2022. godine testirani su PCR-om na SARS-CoV2 putem brisa nazofarinksa, sukladno politikama bolnice u vezi pandemije COVID-19. Unutar 24 sata, za sudjelovanje u studiji pozvani su svi kvalificirani pacijenti (stariji od 18 godina). Izdisani zrak je naknadno prikupljen i analiziran pomoću GC-IMS-a. Prikupljeni podaci statistički su evaluirani koristeći IBM SPSS 25. Primijenjene su deskriptivne statistike, Mann-Whitney U test, chi-kvadrat test i Fisherov egzaktni test, pri čemu je vrijednost p postavljena na <0,05. Kako bi se identificirali značajni VOC-ovi u spektrima GC-IMS-a, korišten je softver temeljen na analizi klastera, a potom multivarijatna statistička analiza.

Rezultati: U ovoj studiji je mjereno i analizirano 89 pacijenata. Od njih, 58 osoba je testirano pozitivno na SARS-CoV-2 s RT-PCR-om i srednjom vrijednošću CT vrijednosti od 25,9. Nisu pronađene značajne razlike između skupina u pogledu dobi i spola. Analiza izdisanog zraka pomoću GC-IMS-a otkrila je 51 klaster, od kojih se 3 činila relevantnima za COVID-19. Rezultati klasifikacije pokazali su da je 76,4% izvornih grupiranih slučajeva ispravno klasificirano, dok je 75,3% križno validiranih grupiranih slučajeva ispravno klasificirano. Osjetljivost se činila 78,9%, a specifičnost 71,9%.

Zaključci: Rezultati ove eksperimentalne studije slučaja s kontrolom pokazuju da se COVID-19, kao primjer zarazne bolesti uzrokovane virusom, može otkriti putem izdisanog zraka pomoću GC-IMS-a. Mjerenje izdisanog zraka i njegova analiza temeljena na sastavu VOC-ova su neinvazivni, brzi (<5 minuta), jeftini, lako izvodljivi i lako dostupni testovi, te predstavljaju obećavajući alat za provođenje skrininga ili prethodnog testiranja na licu mjesta. Međutim, zbog umjerene točnosti u ovoj studiji, potrebne su daljnje eksperimentalne studije na širokoj populaciji kako bi se potvrdila dijagnostička točnost analize izdisanog zraka putem GC-IMS-a.