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Review

MALDI MS-Based Investigations for SARS-CoV-2 Detection

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Abstract: The urgent need to fight the COVID-19 pandemic has impressively stimulated the efforts of the international scientific community, providing an extraordinary wealth of studies. After the sequence of the virus became available in early January 2020, safe and effective vaccines were developed in a time frame much shorter than everybody expected. However, additional studies are required since viral mutations have the potential of facilitating viral transmission, thus reducing the efficacy of developed vaccines. Therefore, improving the current laboratory testing methods and developing new rapid and reliable diagnostic approaches might be useful in managing contact tracing in the fight against both the original SARS-CoV-2 strain and the new, potentially fast-spreading CoV-2 variants. Mass Spectrometry (MS)-based testing methods are being explored, with the challenging promise to overcome the many limitations arising from currently used laboratory testing assays. More specifically, MALDI-MS, since its advent in the mid 1980s, has demonstrated without any doubt the great potential to overcome many unresolved analytical challenges, becoming an effective proteomic tool in several applications, including pathogen identification. With the aim of highlighting the challenges and opportunities that derive from MALDI-based approaches for the detection of SARS-CoV-2 and its variants, we extensively examined the most promising proofs of concept for MALDI studies related to the COVID-19 outbreak.

Keywords: SARS-CoV-2; coronavirus; virus; COVID-19; mass spectrometry; MALDI-TOF; MALDI-FT-ICR; molecular diagnosis; proteotyping; biotyping; genotyping; laboratory testing

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1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic outbreak caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread all over the world, with more than 242 million individuals infected and approximately five million deaths [1].

Recent evidence confirms that in contrast with SARS-CoV-1, which was only transmitted by people with symptoms, the new coronavirus can be transmitted even before the onset of symptoms from pre-symptomatic subjects or even from asymptomatic people. Thus, the silent chains of transmission, which considerably hinder containment efforts aimed at limiting viral spread, require the quick screening of each positive subject in order to help control the ongoing pandemic [2–5].

Currently, together with the use of vaccines, an accurate and immediate diagnosis of SARS-CoV-2 might be one of the most important lines of attack for controlling the spread of infection since it allows for the prompt isolation of people infected by the virus. Additionally, an earlier diagnosis may lead to a more effective therapeutic intervention, with improved prognosis and a higher probability of avoiding patient recovery in the intensive care unit (ICU).

Several SARS-CoV-2 variants have been identified in many countries, which represents one of the most relevant issues in controlling the COVID-19 pandemic [6]. Viral mutations are of great concern as such mutations have the potential to facilitate viral transmission and to reduce the efficacy of developed vaccines by their ability to escape protection due to vaccine-induced neutralizing antibodies [7].

Therefore, it has become necessary to improve current laboratory testing methods and to develop new, rapid, and reliable diagnostic approaches to identify all positive cases and manage contact tracing in order to stop the SARS-CoV-2 and new, potentially fast-spreading SARS-CoV-2 variants.

The development of new diagnostic tools for emerging pathogens requires the ability to detect low viral loads, which enables early detection in order to avoid cross-reactivity with other viral strains and to deliver results quickly.

The clinical diagnosis of suspected cases is generally established by Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR), which represents the gold standard for the molecular diagnosis of SARS-CoV-2. Enzyme-linked immunoassays (EIA) for viral antibody and antigen detection, and serum viral neutralization (SVN) assays for antibody neutralization determination constitute other relevant laboratory tools [8].

However, RT-qPCR may suffer from the lack of sensitivity for the detection of SARS-CoV-2 in the early stages of infection as the concentration level of the virus is low in the upper respiratory airways during the first 6–8 days of illness, only reaching the peak in a window of 10–14 days from the onset of the illness [9,10]. Other limitations of the RT-qPCR assay are its low-throughput capacity due to many intermediate steps and long turnaround times, and the use of a poorly designed specimen extraction control [11,12]. Additionally, false-positive and high false-negative rates ranging from 1% to 30% may arise, depending on several factors such as improper sampling, lack of specificity due to poor sensitivity, and cross- and carryover contamination [13,14]. All together, these factors might lead to misdiagnosis and the spread of infection.

Concerning the rapid antigen tests, these are also being widely adopted for preliminary screening, but they have substantially lower sensitivity than the WHO-recommended standard, especially for pre-symptomatic and asymptomatic cases [15].

Thus, it is of crucial importance in this particular circumstance to develop novel, robust laboratory diagnostic tests that target SARS-CoV-2 with a high level of reproducibility, specificity, and sensitivity [16] to support already existing tests.

Owing to the huge amount of data that can be rapidly collected and considerable technological advancements, mass spectrometry (MS)-based omic approaches have demonstrated, without any doubt, the possibility of performing extensive and sensitive analyses, not only opening new opportunities for the diagnosis and assessment of disease progression, but also providing insights into mechanisms of the disease [17–20].

Additionally, the use of MS platform technologies has already been applied in disease outbreaks for the study and analysis of infectious disease agents such as viruses and bacteria [21–27], suggesting its potential as the new alternative and highly specific test for the SARS-CoV-2 infection.

In particular, recent reviews confirm how MS-based omic technologies have greatly contributed to the detection of SARS-CoV-2 [20,28–31].

Many efforts have been explored to make the MS-detection process high-throughput, highly accurate, and sensitive [32–35].

In this scenario, Matrix-Assisted-Laser-Desorption/Ionization (MALDI)-MS seems to satisfy particularly the high-throughput requirements as well as the rapid data acquisition [36]. In addition, the advancements reached by the Fourier-Transform Ion Cyclotron Resonance (FT-ICR) technology in terms of high and ultra-high-resolution power and mass accuracy in modern mass instruments make possible the development of high-throughput, highly accurate, and highly sensitive processes [37].

MALDI-MS has become one of the main proteomic tools for its relatively high tolerance of mixtures and biological contaminants [36]. This technique is largely used for the

characterization of biomolecules, especially proteins and peptides. However, the original purpose of this technology has also been redirected to microorganism identification. In the last two decades, MALDI has been successfully integrated into the microbiology laboratory workflow due to its capacity for rapid, low-cost microbial species identification, and it is now routinely adopted in Europe and the United States for bacteria identification [38]. Therefore, considering that most clinical diagnostic laboratories already have MALDI-MS equipment, its application in emerging pathogen research as a screening method for COVID-19 detection in large populations presents great potential.

In this paper, we review exploratory MALDI-based approaches for the detection of SARS-CoV-2 and its variants, with the aim of highlighting challenges and opportunities that derive from these laboratory platforms.

2. MALDI-TOF and MALDI-FT-ICR Mass Spectrometry: A Brief Presentation

The discovery of "soft ionization" methods—MALDI and electrospray ionization (ESI)—in the mid-1980s represented a milestone in the history of MS [39,40]. In fact, before the invention of "soft ionization" techniques, some biomolecules decomposed or were destroyed when analyzed by traditional MS instruments. MALDI is a soft ionization technique used in MS that can preserve the structure of large, non-volatile, and labile molecules, allowing for the sensitive detection of many kinds of nonvolatile biomolecules, including proteins, nucleic acids, and carbohydrates [41]. A typical MALDI preparation relies on embedding these biomolecules in an energy-absorbing crystalline matrix (typically, a small aromatic acid). The use of a large excess of matrix over the biomolecules that are of interest for the analysis is at the core of the MALDI principle [41]. More specifically, in a typical experimental procedure (as shown in Figure 1A), the laser beam hits the co-crystallized mixture of the matrix with the analyte. After the matrix absorbs the laser energy, it desorbs from the sample surface, carrying the embedded analytes into the gas phase. In this process, analyte molecules become ions by a proton transfer from the matrix to the analyte. Then, the charged particles, predominantly singly charged ions, are accelerated with a constant amount of kinetic energy to a mass analyzer such as the Time-of-Flight (TOF) analyzer (Figure 1B). While flying through the TOF, the ions are separated according to their mass-to-charge ratio (m/z).

As also illustrated in Figure 1B, in modern MALDI-TOF instruments, sample analysis can be performed using linear and/or reflector MS modes. The linear MS mode is well-suited for analytes with molecular weights above 4000 Da and enables very sensitive analyses. Analytes below 4000 Da are analyzed in reflector MS mode, with improved mass accuracy and resolution. Specifically, when operating in reflector MS mode, a reflector mirror which reflects ions back toward the reflector detector is used. Therefore, a longer flight ion path is provided, which increases ion separation through an enhanced resolution (Figure 1B). The reflector (also used in the MS/MS mode) is a two-stage electrostatic mirror consisting of a series of plates. The reflector also improves mass accuracy and resolution by correcting the dispersion time caused by variations of initial kinetic energy that generate slight differences in ion velocity and by filtering out neutral molecules. In the presence of a second TOF analyzer (MALDI-TOF/TOF), samples analyzed in the reflector MS mode may be further analyzed by the MS/MS mode to obtain a fragmentation pattern used to determine structural information. This platform is especially used in peptide/protein identification and amino acid sequencing. MALDI-TOF MS allows for the analysis of hundreds of proteins or other biomolecules in a single spectrum and has been used in several diagnostic applications in recent years, including: (i) the study of the distributions of particular biochemical compounds on tissues (MALDI-based imaging MS) [42,43]; (ii) the analysis of nucleic acids for genotyping single nucleotide polymorphisms (SNPs) [44]; (iii) the profiling of biological samples to aid in the discovery of new of disease biomarkers in exploratory studies with diagnostic applicability. Such biomarkers might be used as indicators of progress or the severity of diseases/disorders [45–51], and for (iv) the identification, characterization, and typing of bacteria, yeasts, and viruses [52–56].

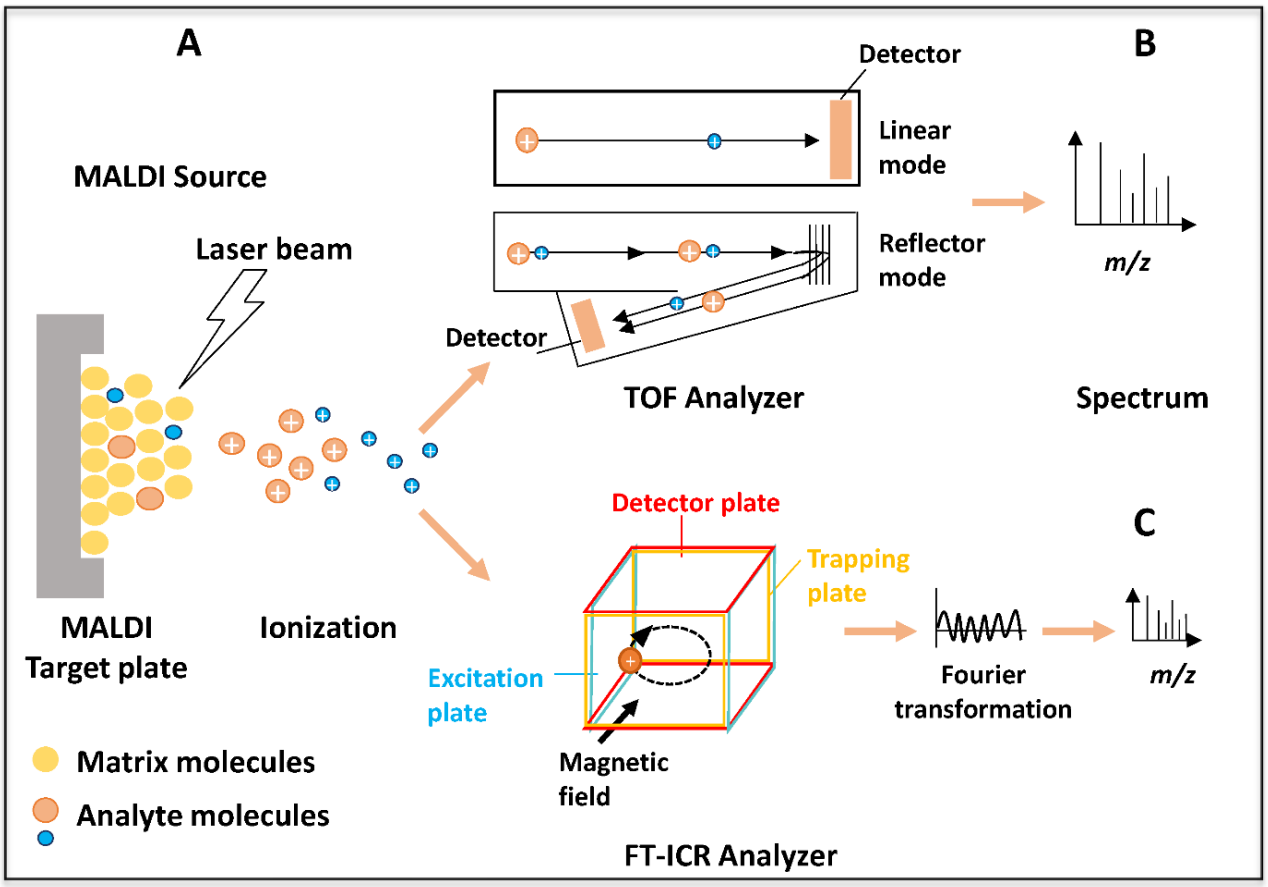

Figure 1. Schematics of the MALDI-MS preparation and the equipment used for SARS-CoV-2 detection. In the sample preparation step, analytes are mixed with a large excess of matrix and are spotted onto a stainless steel target plate, where they co-crystallize. In the MALDI source, (**A**) the co-crystals are irradiated with a laser beam, inducing the desorption and ionization of analytes. The generated ions are accelerated in an electric field, which directs them to the analyzer. In the TOF analyzer, (**B**) ions are separated according to their m/z in a flight tube by two different modes: linear and reflectron. The ions are accelerated toward a detector, which amplifies ion signals and converts them into a mass spectrum. In the FT-ICR analyzer, (**C**) the m/z are measured according to the cyclotron frequency of the ions, trapped in a circular orbit, and subjected to a magnetic field, subsequently generating a signal. Using a Fourier transform, the signal is converted into a mass spectrum. The MALDI mass spectrum represents the mass-to-charge ratios of each ionized molecular species in a sample.

Although less used than TOF because of its high costs, another mass analyzer coupled with a MALDI source is the FT-ICR, which provides better performance than a MALDI-TOF instrument in terms of resolution, mass accuracy, and dynamic range. The FT-ICR analyzer measures the mass-to-charge ratio (m/z) of ions according to the cyclotron frequency of the ions (Figure 1C). After ion generation in the MALDI source, the ions are transferred to the Penning trap consisting of a cell under a high vacuum positioned in a superconducting magnet. The ions are excited by a radiofrequency and generate a signal. The frequency of the signal from each ion is equal to its orbital frequency, which is inversely related to its m/z value. The signal intensity of each frequency is proportional to the number of ions with that m/z value. The signal is amplified and all the frequency components are determined. The mass spectrum is generated using Fourier analysis (Figure 1C). The frequencies match the m/z, and the amplitudes are correlated with the abundance of the analytes [57]. In comparison to any other mass analyzer, FT-ICR provides the highest mass accuracy and mass resolving power, reaching up to ppb mass accuracy, high dynamic range, and mass resolving power greater than 1,000,000 in routine analyses [58]. Very recently, molecular structures with m/z differences of 1.79 mDa were resolved and identified with

high mass accuracy and sensitivity [37]. For these reasons, MALDI-FT-ICR is very suitable for the analysis of complex biological samples in proteomic, metabolomic, and lipidomic research in different fields of application, including microbiology, imaging, and biomarker discovery [59–61].

3. MALDI-MS for Pathogen Detection: A General Overview

Over the past decade, MALDI-MS has become a routine laboratory technique for the rapid, accurate, and cost-effective identification of cultured bacteria and fungi in clinical microbiology, which requires minimal consumables or reagents [62–64]. MALDI-TOF technology has been used for the study, and analyses of several viruses such as human herpesviruses, influenza viruses, and diseases that are related to severe enterovirus infections such as echovirus, coxsackievirus A and B, and poliovirus [53,54,65–68]. Moreover, the advantage of this approach over the low-throughput LC-MS-based methods is the wide availability of this instrumentation in clinical laboratories, including those in developing countries; consequently, the implementation of the developed methodology would not require a high economic cost [62].

MALDI-MS approaches used for SARS-CoV-2 detection are illustrated in Figure 2. As exemplified in Figure 2, current MALDI-MS approaches aimed at the detection of SARS-CoV-2 are based on "biotyping" [69,70] and "genotyping" [71–73] strategies. One study is based on "proteotyping" with high resolution MALDI-FTICR at the peptide level [74], while several studies used the "biomolecular host profiling" strategy to uncover biomarkers of diagnostic utility generated after SARS-CoV-2 infection [75–79]. In particular, the investigations by Iles et al. and Chivte et al. used both biotyping and biomolecular host profiling strategies [69,70].

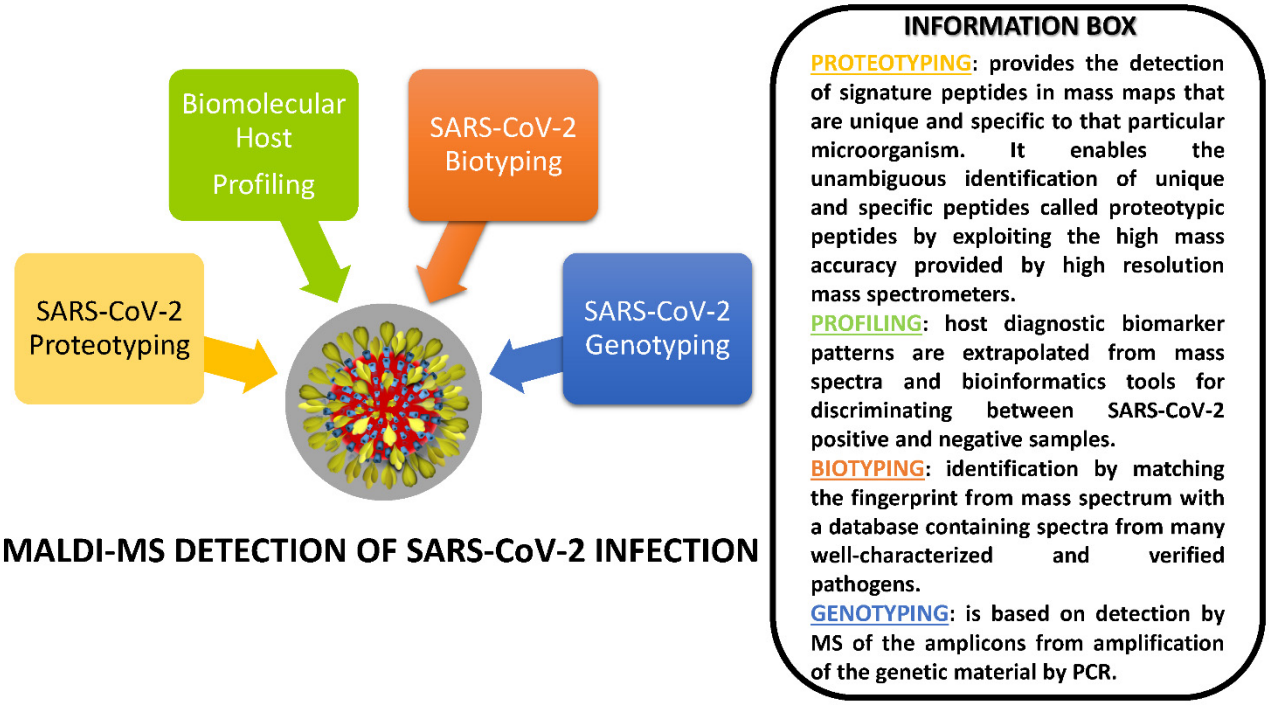

Figure 2. Overview of the MALDI-MS approaches for SARS-CoV-2 detection. MALDI-MS biotyping, proteotyping, and genotyping provide the direct detection of SARS-CoV-2. MALDI-MS biomolecular host profiling approaches uncover biomarkers associated with COVID-19.

The identification of microorganisms by MS is a relatively recent concept that is different from the traditional typing based on DNA restriction or amplification methods.

Specifically, proteins are extracted and profiled by MALDI-TOF MS to generate the fingerprint that is used to identify the microorganisms. However, it is difficult and very

challenging to perform the MS-biotyping analysis with viruses as they have low protein content in general. The MS-biotyping procedure is mainly used for bacteria, yeasts, and molds. This "biotyping" procedure allows for the identification of a strain by matching its fingerprint obtained from a mass spectrum with a database containing spectra from many well-characterized and verified pathogens. The success of MALDI biotyping mostly resides in the shorter time it requires to produce results in comparison to classical genotypic or phenotypic identification. While accurate identification is most reliably accomplished at the species level, limitations of the method may arise when differentiating closely related species. However, to overcome these limitations, tandem MS approaches at the peptide level have been employed [80–82]. The tryptic digestion of the extracted proteins from the classical biotyping preparation in combination with nano-LC and the subsequent identification of the peptides by tandem MS increases the discrimination power at the subspecies level [83]. The term "proteotyping" refers predominantly to proteomic bottom-up approaches. "Proteotyping" strategies mainly use single signature peptides detected in mass maps to identify, type, and subtype strains, using high-resolution MS. More specifically, MS proteotyping facilitates the detection of signature peptides in mass maps that are unique and specific to that particular microorganism. This approach enables the unambiguous identification of these unique and specific peptides (proteotypic peptides), exploiting the high mass accuracy provided by high resolution mass spectrometers [84]. Proteotyping approaches have been used in the identification of different viruses such as the influenza virus [84], the parainfluenza virus [85], and the oncovirus [86], and for discerning different biopathogen species as well as strains within the same species. High-resolution MALDI-based approaches are the MS instruments most suited to proteotyping; in addition to the simplicity of their sample preparation, these approaches employ high-throughput processes and automation.

MS-based genotyping methods are based on the detection by MS (especially MALDI-TOF MS) of the amplicons from the PCR amplification of the genetic material. The genotyping approaches exploiting the MS detection of PCR amplicons are very sensitive; consequently, enrichment methods such as cell culturing can be avoided. These approaches have been used successfully for the identification of several viruses and bacteria [71–73,87–89].

Very recently, low-cost panels enabling the accurate, high-throughput, and low-cost detection of SARS-CoV-2, dominant SARS-CoV-2 variants, and influenza RNA have been developed by Agena Bioscience® (San Diego, CA, USA). In particular, the MassARRAY SARS-CoV-2 Panel (EUA, CE-IVD) [90] will be briefly described in the next sections.

In contrast to the previously described approaches, which detect viral proteins or viral genes directly and unambiguously, the biomolecular host profiling approaches instead measure changes in host biomolecular MS profiling. These studies are very challenging as a large and stratified cohort population is required. Moreover, disease assessments to verify the presence of other comorbidities are mandatory in order to better elucidate MS profiling readouts and avoid confounding the results [91,92].

Table 1 mainly summarizes all the MALDI-MS-based SARS-CoV-2 detection studies: the specimen, experimental strategies, MS-targeted molecules, and other critical details such as the time necessary to complete the whole assay and the diagnostic performances such as sensitivity and specificity.

The general workflow of all these investigations is illustrated in Figure 3. Control subjects and (PCR-diagnosed) SARS-CoV-2 patients were enrolled; different sampling procedures were used to collect samples. Subsequently, depending on the MALDI-MS approaches used (proteotyping, biotyping, genotyping, and biomolecular host profiling), the extraction of biomolecules (RNA or proteins) was performed. Samples were subsequently subjected to MALDI-MS analysis, and finally, the resulting data were analyzed using online or in-house database searching or machine learning algorithms, thus allowing for the detection of SARS-CoV-2 infection.

Table 1. MALDI-MS-based SARS-CoV-2 detection studies: specimen, experimental strategy, target molecules, and diagnostic performances.

Specimen	Sample Size	MS Instrumentation	Identification Method	Molecular Target	Use of Database or Algorithms for Identification	Time	Diagnostic Performances	References
Nasopharyngeal samples	Not specified	MALDI-FT-ICR	Proteotyping	Viral proteins: - N (1); - S (2); - M (3).	Online database searching	Similar to RT-PCR time frame (few minutes for mass spectra acquisition)	Only analytical performances	Dollman et al. [74]
Saliva or gargle samples	35 samples	MALDI-TOF MS	Biotyping and Biomolecular Host Profiling	Host proteins; viral proteins: - S; - Veps (4).	Output data processed with appropriate software (not specified)	45 min for sample preparation, 3 min per sample for MALDI-TOF analysis, a few seconds for data results analysis	Sensitivity of ~100% (5)	Iles et al. [69]
Gargle samples	- 30 COVID-19 positive samples (89% asymptomatic) - 30 COVID-19 negative samples	MALDI-TOF MS	Biotyping and Biomolecular Host Profiling	Host proteins; viral proteins: - S.	Online database searching	Not mentioned	- Sensitivity of 93.33–100% - Specificity of 90–93.33% (6)	Chivte et al. [70]
Oral or nasopharyngeal samples	- 22 COVID-19 positive samples - 22 COVID-19 negative samples	MALDI-TOF MS	Genotyping	Viral genes: - N1; - N2; - N3; - ORF1 (7); - ORF1ab.	Not specified	8 h for the entire process	Not mentioned	Wandernoth et al. [71]
Oral or nasopharyngeal samples	168 suspected COVID-19 samples	MALDI-TOF MS	Genotyping	Viral genes: - N1; - N2; - N3; - ORF1; - ORF1ab.	Online database searching	8 h for the entire process	Not mentioned	Rybicka et al. [72]
Saliva samples	- 34 COVID-19 positive samples - 26 COVID-19 negative samples	MALDI-TOF MS	Genotyping	Viral genes: - N1; - N2; - N3; - ORF1; - Orf1ab.	Not specified	Not mentioned	- Sensitivity of 97.14% - Specificity of 100% (8)	Hernandez et al. [73]
Nasopharyngeal samples	- 211 COVID-19 positive samples - 151 COVID-19 negative samples	MALDI-TOF MS	Biomolecular Host Profiling	Host proteins	Machine learning algorithms	Not mentioned	- Sensitivity of 94.7% - Specificity of 92.6% (9)	Nachtigall et al. [75]

Table 1. *Cont.*

Specimen	Sample Size	MS Instrumentation	Identification Method	Molecular Target	Use of Database or Algorithms for Identification	Time	Diagnostic Performances	References
Nasal samples	- 107 COVID-19 positive samples (28 asymptomatic and 79 symptomatic) - 92 COVID-19 negative samples	MALDI-TOF MS	Biomolecular Host Profiling	Host proteins	Machine learning algorithms	Total turnaround time < 1 h	- Accuracy of 98.3%, PPA [10] of 100%, NPA [11] of 96% for DNN [12] model - Accuracy of 96.6%, PPA of 98.5%, and NPA of 94% for GBM [13] model [9]	Tran et al. [76]
Nasopharyngeal samples	237 samples	MALDI-TOF MS	Biomolecular Host Profiling	Host proteins	Machine learning algorithms	Turnaround time < 2 h	- Sensitivity of 100% - Specificity of 92% - Accuracy of 97% [9]	Deulofeu et al. [77]
Nasopharyngeal samples	311 samples	MALDI-TOF MS	Biomolecular Host Profiling	Host proteins	In-house database searching and machine learning algorithms	Not mentioned	- Sensitivity of 61.76% - Specificity of 71.72% - Accuracy of 67.66% [9]	Rocca et al. [78]
Serum samples	- 146 COVID-19 positive samples - 152 COVID-19 negative samples	MALDI-TOF MS	Biomolecular Host Profiling	Host proteins	Machine learning algorithms	Less than 1 min per sample for MALDI-TOF analysis	- Sensitivity of 98% - Specificity of 100% - Accuracy of 99% [9]	Yan et al. [79]

[1] N: nucleocapsid protein; [2] S: spike protein; [3] M: membrane protein; [4] Veps: viral envelope proteins; [5] Diagnostic sensitivity of the method is reported; [6] Sensitivity and specificity are referred to AUC and ROC curve analyses; [7] ORF: open reading frame; [8] Percent agreement and Cohen's kappa were calculated to assess sensitivity and specificity; [9] Diagnostic performances are referred to the Machine learning model used. [10] PPA: positive percent agreement; [11] NPA: negative percent agreement; [12] DNN: deep neural network; [13] GBM: XGBoost gradient boosting machine.

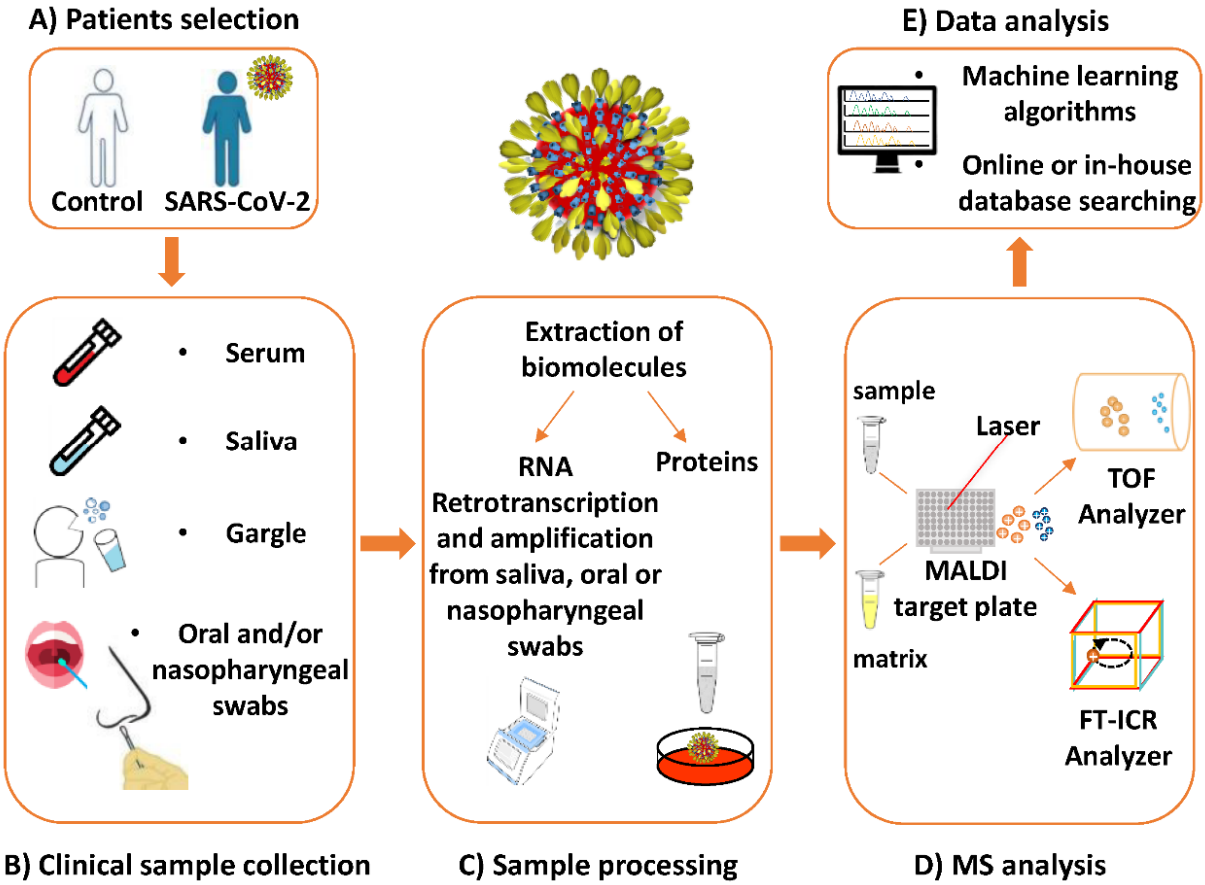

Figure 3. Workflow of main MALDI MS-based investigations targeting SARS-CoV-2. (**A**) Control subjects and (PCR-diagnosed) SARS-CoV-2 patient enrollment. (**B**) Clinical sample (serum, saliva, gargle, oral, and/or nasopharyngeal swab) collection from control subjects and SARS-CoV-2 patients. (**C**) Sample processing based on the extraction of RNA or proteins. RNAs are retro-transcripted and amplified from saliva, oral, and/or nasopharyngeal swabs in genotyping methods; proteins are extracted from body fluid samples or from cell-cultured SARS-CoV-2 samples in the proteotyping and biotyping methods; in the case of biomolecular host profiling methods, proteins are extracted from bodily fluids. (**D**) MS analysis based on the ionization in a MALDI source and the separation of the ions into two different types of analyzers: TOF and FT-ICR. (**E**) Data analysis using online or in-house database searching or machine learning algorithms for the detection of SARS-CoV-2 infection.

4. MALDI-MS Investigations Targeting SARS-CoV-2

In this section, we report the MALDI-MS-based investigations which aimed to detect SARS-CoV-2 infection with the use of "proteotyping" [74], "biotyping" [69,70], "genotyping" [71–73], and biomolecular host profiling [75–79] approaches. Interestingly, two of these investigations used both biotyping and biomolecular host profiling strategies [69,70].

4.1. Proteotyping Approach for SARS-CoV-2 Detection by MALDI-MS

Dollman and colleagues applied a proteotyping strategy for the detection of peptides unique to the SARS-CoV-2 by mass alone; they used High Resolution MALDI-FT-ICR Mass Spectrometry [74]. In particular, nasopharyngeal (NP) swabs from infected patients were cultured in Vero E6 cells or directly prepared before the MALDI-MS analysis. The use of a virus culture provided a more reliable approach to overcome potential false negatives deriving from specimens with low viral loads. The authors performed the MALDI-FT-ICR-MS analyses on the tryptic whole virus digest peptide products. In the case of cell-cultured SARS-CoV-2 samples with higher virus titers in comparison to other samples, twenty of the

most prominent signals were confidently matched to fragments of the N, M, and S proteins with a high mass accuracy (<3 ppm) (Table 1). Detected peptides covered a large range of identified N- and C-terminal, fusion peptide receptor, and RNA-binding domains.

The acquired mass spectra from the non-cell-cultured NP samples showed peptide ions with reduced intensity and lower signal-to-noise ratio (S/N) as compared to those from experiments using cell-cultured samples; in this case, additional matrix cluster ions were also observed. However, despite the lower virus titers, the tryptic peptides of M, N, and S proteins were detected, including a new peptide at m/z 843.4255. In particular, six peptides were always detected in ten different NP samples, and five represent reliable signature peptides for SARS-CoV-2 proteotyping. In fact, due to the shared clinical symptoms presented by the SARS-CoV-2 with other circulating influenza viruses, the authors, by means of a specifically constructed algorithm and database, showed that these peptides were detected in all SARS-CoV-2 strains and exhibited a difference in mass greater than 3 ppm in comparison to those fragments detected in more than 95% of the cases from all influenza virus proteins of the circulating strains.

Table 2 reports the limit of detection (LOD) of the various analytical assays (including LOD related to conventional RT-PCR [93]) calculated based on the information from each of the authors, in order to make the comparison more straightforward for the readers. To determine the LOD of this MS platform, serial dilution experiments on the cell-cultured sample were performed, and the most abundant viral peptide signal at m/z 1635.8238 was monitored by MS. The peak was detected down to 0.75 ng, matching 7.5×10^5 copies (Table 2).

This represents an excellent result, considering that a recent study reported maximum estimated levels of virus collected by NP swabs varying from 10^6 to 10^9 copies [94].

Interestingly, the authors stressed that this copy number is comparable with the 10^5–10^6 copies of virus necessary for high-quality PCR sequencing; however, as they additionally claimed, it is also higher than the 10^3–10^4 copies usually required for virus detection by PCR (Table 2). As underlined by the authors, the detection limits of 10^5 copies could be significantly improved when operating in an automated selected ion monitoring (SIM) strategy for the detection of only selected peptides for a proteotyping assay (Table 2). Furthermore, this MS approach does not require the separation of viral components, thus allowing for mass spectra acquisition from hundreds of digested virus samples in only a few minutes. The use of immobilized enzymes for proteolytic digestion as well as the implementation of an automated or semi-automated spectral acquisition method could drastically reduce processing and acquisition time analysis. Other advantages include better speed of the analysis as compared to LC-MS approaches and the possibility to confidently assign viral peptides by mass alone, without the use of MS/MS sequencing, which further reduces the time required for the analysis. For all these reasons, MS virus proteotyping should play a key role in molecular detection, not only for SARS-CoV-2 but also for other respiratory viruses and bio-pathogens. The high costs of the MS equipment, due to the high-resolution FT-ICR analyzer, make this MS-based proteotyping assay too expensive, although their costs are comparable to sequencers and PCR tools, as the same authors observed.

Table 2. Diagnostic performances and estimated costs of the different tests used for SARS-CoV-2 detection.

Specimen	Test Category	LOD [(1)]	Analysis Time	Estimated Cost per Sample	Key Points	References
Respiratory tract specimens	RT-PCR	<10 copies/reactions (10^3–10^4 copies) [(2)]	4–6 h	USD 10–15	Current gold standard for COVID-19 diagnosis.	Kevadiya et al. [93]
Nasopharyngeal samples	MALDI-FT-ICR	10^5 copies	Similar to RT-PCR time frame	USD 100	LOD could be improved in automated selected ion monitoring (SIM) strategy (reaching 10^3–10^4 copies).	Dollman et al. [74]
Saliva or gargle samples	MALDI-TOF MS	~10–10^2 copies	~50 min	Less than USD 1	Specificity at 10–10^2 copies was reached only for the S1 protein peak.	Iles et al. [69]
Gargle samples	MALDI-TOF MS	~30 copies	Not mentioned	Not mentioned	MS protocol was sensitive and comparable with RT-PCR forlow viral loads.	Chivte et al. [70]
Oral or nasopharyngeal samples	RT-PCR/MALDI-TOF MS	~10 copies	8 h	Not mentioned	Time-to-results was faster for RT-PCR, while hands-on time was comparable between RT-PCR and MS assay techniques.	Wandernoth et al. [71]
Oral or nasopharyngeal samples	RT-PCR/MALDI-TOF MS	~10 copies	8 h	~ EUR 10	The MS assay was able to detect SARS-CoV-2 in low viral load specimens.	Rybicka et al. [72]
Saliva samples	RT-PCR/MALDI-TOF MS	~10^3 copies	Not mentioned	Not mentioned	The LOD of 10^3 copies was obtained for the N2 target.	Hernandez et al. [73]

[(1)] LOD: limit of detection; [(2)] 10^3–10^4 copies are typically required for PCR detection [74].

4.2. Biotyping Approach for SARS-CoV-2 Detection by MALDI-MS

With the aim of carefully and systematically analyzing all the challenging issues of an MS-based diagnostic process, Iles and colleagues developed a clinical MALDI-TOF MS assay for SARS-CoV-2 detection in a saliva or gargle solution [69]. The samples needed to be deactivated by UV-C irradiation, which preserves the viral membrane and proteins necessary for MS analysis. On the contrary, the use of heat for inactivating SARS-CoV-2 in NP samples as well as the use of SDS or Triton media for storage and transport could strongly interfere with MS analysis. Moreover, in comparison to NP or oropharyngeal swabs (OP), the saliva or gargle solution could be collected without causing as much discomfort to individuals. The authors performed several experiments in order to optimize the MALDI-TOF detection of viral protein and its proteolytic peptide detection. They finally developed a clinical diagnostic protocol based on (1) the collection of gargle/saliva samples; (2) the rapid processing of the gargle samples by filtration, acetone precipitation with subsequent pellet resuspension, and protein solubilization; (3) MALDI TOF analysis; (4) output data processing by an appropriate software (see Table 1).

The authors found that the use of ice-cold acetone for viral particle enrichment by precipitation was more advantageous over other organic solvents. The precipitation, followed by centrifugation, allowed for the enrichment of the pellet, with the enrichment of large virus particles and the removal of unwanted background proteins contained in the supernatant. Moreover, the use of ice-cold acetone also strongly contributed to virus inactivation.

Sinapinic acid (SA) was found to be more sensitive for the detection of virus-specific glycoproteins in comparison to alpha-cyano-4-hydroxycinnamic acid (CHCA), which is used for the routine detection of microbial intracellular proteins. Low resolution MALDI-TOF spectra were acquired in which S-protein subunits with their fragments, S1 (at ~m/z 79,000) and S2 (m/z from ~62,000 to ~72,000), were detected together with other putatively identified viral envelope protein fragments (m/z from ~26,000 to ~47,000). S1 peaks were higher in the SARS-CoV-2 virus culture spiked samples than in the volunteer samples, demonstrating that this marker alone could be an indicator of the identification of the coronavirus, with nearly 100% detection and specificity at 10^3–10^4 PFU SARS-CoV-2 virions in a gargle/saliva sample, which correspond to 10–10^2 copies (Table 2).

Interestingly, through the clinical information extrapolated by MALDI-TOF analysis, the authors demonstrated not only the possibility to detect S protein fragments, but also other peaks matching with Immunoglobulin light chains indicative of an oral upper respiratory immune response, and elevated levels of gargle/saliva IgA heavy chain peak indicative of a viral immune response.

These features reveal the diagnostic utility of MALDI-TOF MS as a powerful and economically ideal solution, with its ease of sampling and speed of analysis. However, other studies and validation tests on saliva/gargle spiked with cultured virus, or a direct comparison of the MALDI-TOF MS analysis with an RT-PCR detection of COVID-19 in clinical samples is required.

Starting from the results obtained by the study of Iles and coworkers, Chivte and colleagues analyzed gargle samples with MALDI-TOF MS to assess the presence of SARS-CoV-2 infection and compared the resulting spectra with the corresponding results of an RT-qPCR from NP swabs [70]. In particular, they analyzed 60 gargle samples (see Table 1) from volunteer student athletes, including 30 PCR positive and 30 PCR negative samples. Spectral clear differences were observed in the m/z range from 20,000 to 200,000. This extended m/z range allowed them to analyze the area under the curve (AUC) of putative viral and host proteins in order to assess the presence of SARS-CoV-2 infection. The receiver operating characteristic (ROC) analysis identified five peaks which showed highly sensitive and specific discrimination between COVID-19 positive and negative samples, including peaks which probably corresponded to the S1 and S2 fragments of the SARS-CoV-2 S protein, a potential biomarker near m/z 112,000, and peaks putatively assigned as human immunoglobulins or human α-amylase (Tables 1 and 3). The putative identification

of these peaks was assessed through a comparison with the UNIPROT database and from the work of Iles et al.; however, these results need to be further validated. For these biomarkers, an elevated concordance was achieved (90%) with the RT-PCR results, demonstrating that this methodology is a promising tool for a rapid and inexpensive COVID-19 assay. To evaluate the LOD of the MALDI-TOF protocol, they analyzed a saliva sample with a very low SARS-CoV-2 viral load by quantitative RT-qPCR. In particular, the Ct value of this sample was 36.09, which is less than the quantifiable limit of the assay (~30 copies) (Table 2). Interestingly, the authors observed a signal in the mass spectrum for the potential biomarker peak found between 78,600 and 80,500 m/z, demonstrating that the MALDI-TOF protocol could detect SARS-CoV-2 in samples containing very low viral loads.

4.3. Genotyping Approach for SARS-CoV-2 Detection by MALDI-MS

Wandernoth and colleagues evaluated the ability of MALDI-TOF MS to detect nucleic acid from SARS-CoV-2 in biological samples and compared the results to those of rRT-PCR [71]. In particular, they analyzed oral and NP swab samples from 22 patients who tested positive and 22 patients who tested negative by RT-PCR for SARS-CoV-2 infection (see Table 1). They used the new commercially available MS-based assay MassARRAY® (Agena Bioscience®, San Diego, CA, USA), a genotyping panel for the detection of SARS-CoV-2 developed by Agena Bioscience, which received the CE-IVD mark in Europe for the detection of nucleic acid from SARS-CoV-2 in respiratory specimens. This platform provides a robust route for the detection of the SARS-CoV-2 virus in human samples. It consists in a four-step process represented by a one-step RT-PCR reaction to reverse transcribe viral RNA into cDNA, followed by the amplification of the obtained nucleic acid material, primer extension, and the MALDI-TOF analysis of the products on a matrix-loaded silicon chip array. It allowed for the detection of five SARS-CoV-2 specific targets: three in the nucleocapsid region and two in the ORF1ab region. The results of the rRT-PCR and MALDI-TOF MS analyses were comparable in all samples. The LOD was estimated at 10 genome copies (Table 2). Interestingly, time-to-results was faster for rRT-PCR, while hands-on time was comparable between the rRT-PCR and MALDI-TOF techniques. Very recently, Rybicka and colleagues [72] also tested the new commercially available MS-based assay MassARRAY® SARS-CoV-2 Panel (Agena Bioscience) and compared it to the RT-PCR diagnostic test. Oral and NP swabs from 168 suspected COVID-19 patients with symptoms of respiratory infection were simultaneously processed with both assays (Table 1). Among the 168 analyzed samples, different results were obtained for 10.12% (17 samples). In particular, in four samples considered negative by RT-PCR, viral genes were detected by the MS-based method. Furthermore, 87% of patients previously considered as suspected cases by RT-PCR resulted unambiguously positive in all MS assays. An estimated LOD of the assay was 10 copies (Table 2). Using the MassARRAY® SARS-CoV-2 Panel, the authors were able to detect SARS-CoV-2 in low viral load specimens and with very few microliters of viral RNA. The authors emphasized that their data analysis is in agreement with other studies, reporting false-negative results from RT-PCR of about 30%, and that MALDI-TOF MS seems to be an ideal tool for the detection and discrimination of mutations. They pointed out that the MassARRAY® System provides automation, minimal hands-on time, and onboard data analysis, delivering easy-to-interpret data with a simple and fast workflow.

Study limitations may arise from the lack of information about isolated RNA concentration as well as from the inability to reanalyze suspected COVID-19 patients in order to evaluate the progress of infection.

Table 3. Sample size, protein patterns, and their expression trend in COVID-19 vs. non-COVID-19 samples in Biomolecular Host Profiling studies.

Specimen	Sample Size	Patient Classification	Protein/Peptide Identity	*m/z*	Expression (Downregulated ↓, Upregulated ↑) Against Control	Bioinformatic Tool	Sensitivity/ Specificity of ML Diagnostic	References
Gargle	60 samples	- 30 COVID-19 positive samples (89% asymptomatic) - 30 COVID-19 negative samples	Immunoglobulin heavy chain or amylase	55,500–59,000	↑	- AUC [1] - ROC [2]	ML [3] not applied.	Chivte et al. [70]
			Immunoglobulin heavy chain doubly charged	27,900–29,400	↑			
			Not identified	~112,000	↑			
Nasopharyngeal	362 samples	- 211 COVID-19 positive samples - 151 COVID-19 negative samples	Not identified	3358	↓	- ML (SVM-R) [4] - PCA [5] - ROC	- Sensitivity of 94.7% - Specificity of 92.6%	Nachtigall et al. [75]
				3095	↓			
				4532	↑			
				3337	↓			
				3152	↑			
				10,444	↓			
				7612	↓			
Nasal	199 samples	- 107 COVID-19 positive samples (28 asymptomatic and 79 symptomatic) - 92 COVID-19 negative samples	Not identified	Not specified	Not specified	- ML (DNN [6] and GBM [7]) - PCA - AUC - ROC	- Accuracy of 98.3%, PPA [8] of 100%, NPA [9] of 96% for DNN model - Accuracy of 96.6%, PPA of 98.5%, NPA of 94% for GBM model	Tran et al. [76]
Nasopharyngeal	237 samples	Not mentioned	Not identified	Not specified	Not specified	- ML (SVM) - PCA	- Sensitivity of 100% - Specificity of 92%	Deulofeu et al. [77]
Nasopharyngeal	311 samples	Not mentioned	Not identified	3372	↓	- ML - AUC - ROC	- Sensitivity of 61.76% - Specificity of 71.72%	Rocca et al. [78]
				3442	↓			
				3465	↓			
				3488	↓			
				6347	↓			
				10,836	↓			

Table 3. *Cont.*

Specimen	Sample Size	Patient Classification	Protein/Peptide Identity	*m*/*z*	Expression (Downregulated ↓, Upregulated ↑) Against Control	Bioinformatic Tool	Sensitivity/ Specificity of ML Diagnostic	References
Serum	298 samples	- 146 COVID-19 positive samples - 152 COVID-19 negative samples	Not identified	6357	↓	- ML (LR) [10] - AUC - ROC - PCA	- Sensitivity of 98% - Specificity of 100%	Yan et al. [79]
				6654	↓			
				6639	↓			
				28,232	↓			
			Platelet basic protein	13,886	↓			
			Platelet factor 4 variant	7614	↑			
			Hemoglobin subunit alpha	15,123	↑			
			Hemoglobin subunit beta	15,867	↑			
			WD repeat-containing protein	28,091	↑			

[1] Area under the receiver operating characteristic (ROC) curve; [2] Receiver operating characteristic; [3] Machine learning; [4] Support vector machine with a radial kernel; [5] Principal component analysis; [6] DNN: deep neural network; [7] GBM: XGBoost gradient boosting machine; [8] PPA: positive percent agreement; [9] NPA: negative percent agreement; [10] Logistic regression.

Hernandez and colleagues evaluated the ability of two different technologies to detect SARS-CoV-2 in saliva samples. In particular, they compared the already mentioned RT-PCR/MALDI-TOF MS-based assay (AGENA MASS ARRAY) with the cobas® 6800/8800 SARS-CoV-2 real-time RT-PCR (Roche, Basel, Switzerland) for conventional real time RT-PCR [73]. They collected saliva samples from 60 patients, which were previously subjected to molecular testing for SARS-CoV-2 in NP or anterior nare specimens. The Agena MassARRAY® platform allowed for the detection of viral targets: three in the nucleocapsid (N) gene (N1, N2, N3) and two in the Orf1ab gene (ORF1, Orf1ab) (Table 1). However, only two targets were detected by using the cobas® 6800/8800 SARS-CoV-2 real-time RT-PCR (Roche): the SARS-CoV-2-specific Orf1ab gene (T1) and the pan-Sarbecovirus envelope E gene (T2).

Interestingly, SARS-CoV-2 detection in saliva samples by the AGENA system showed high sensitivity and specificity (Table 1) when compared to RT-PCR results from NP or anterior nare specimens. Analogue sensitivity and specificity were obtained by cobas® 6800/8800 SARS-CoV-2 real-time RT-PCR (Roche). Among the Agena targets, the most sensitive were the N2 target (10^3 copies) (Table 2), followed by the N1 target.

The Roche platform showed a higher sensitivity in comparison to the Agena platform, having achieved a lower LOD (390.6 copies/mL).

These data demonstrate that saliva constitutes an appropriate matrix for SARS-CoV-2 detection on the novel Agena system, with a comparable performance to the more ubiquitous real-time RT-PCR technology. As stated by the same authors, one limitation of this study is the lack of a standardized collection method, because saliva samples were collected randomly within two days of initial NP and anterior nare collection, and not at one time point.

4.4. Biomolecular Host Profiling Approach for SARS-CoV-2 Detection by MALDI-MS

In this section, we report on MALDI-MS studies performed on a variety of biological specimens in which host diagnostic biomarker patterns had been extrapolated rather than direct SARS-CoV-2 detection [75–79]. The investigations by Iles and colleagues [69] and by Chivte and colleagues [70], which assessed the presence of both viral and host proteins, were already reported in the previous section (see Tables 1 and 3). Many of these investigations used oral or respiratory samples [75–78], which are the commonly accepted specimens for SARS-CoV-2 detection; interestingly, one such study was conducted using serum samples to decipher the molecular changes induced by the systemic effects after infection [79]. In these investigations, MALDI-TOF readouts were processed by machine learning (ML) techniques (Table 1) to extrapolate novel or unknown diagnostic information from a mass spectra dataset. Additionally, the robustness and clinical significance of the ML models employed were assessed.

Nachtigall et al. described a method of detecting SARS-CoV-2 in NP swabs using the MALDI-TOF MS and ML analyses [75]. They proposed this approach starting from the rationale that several clinical laboratories use MALDI-MS as a high-throughput, cost-effective, and robust technology for the conventional diagnosis of pathogen infections.

They analyzed a total of 362 NP swab samples, 211 RT-PCR positives and 151 RT-PCR negatives, from subjects coming from three different countries (Argentina, Chile, and Peru) (Table 1). The biological samples were placed on the MALDI target plate without prior sample purification.

They established a mass analysis range between 3 and 15.5 kDa and applied a two-tailed Wilcoxon rank sum test, allowing for the identification of 31 m/z peaks that could differentiate SARS-CoV-2 positive from negative samples (none of these were identified). Seven of these were the most relevant peaks at m/z 3095, 3152, 3337, 3358, 4532, 7612, and 10,444 (in particular, the peak at m/z 7612 was common to all the samples from the different laboratories) (see also Table 3).

Extracted m/z features that discriminate between SARS-CoV-2 positive and negative samples were then used for principal component analysis (PCA). The PCA provided only

partial data separation when samples from the three different countries were analyzed all together; on the contrary, complete data separation was achieved on samples from each country independently. Therefore, more advanced methods are needed to better discriminate SARS-CoV-2-positive and negative samples.

Next, the authors tested different feature selection methods and ML approaches to determine the top performing analysis strategies. Feature selection methods can be used in data pre-processing to obtain efficient data reduction, a useful step in order to identify accurate data models [95]. In particular, they applied feature selection methods with six different ML algorithms in a smaller set of 80 SARS-CoV-2-positive and negative samples. The resulting data demonstrated that although the support vector machine model (SVM-R) with no feature selection was the best method for SARS-CoV-2 detection, model accuracy did not vary substantially among the ML methods. Finally, Nachtigall and colleagues tested the SVM-R ML algorithm, with and without the feature selection methods, on all 362 samples from all three countries. This ML algorithm with no feature selection method allowed for the discrimination of the control group from patients with COVID-19 and reached high sensitivity and specificity levels (Table 1). Through a comparison of the results obtained from RT–PCR and MALDI-TOF coupled with ML, the authors found a concordance rate that was acceptable as a clinical diagnostic approach (>80%), confirming that the MALDI-MS and ML analyses represent promising alternatives as fast screening assays for SARS-CoV-2.

Tran and colleagues described as proof-of-concept a novel, automated, ML-enhanced MALDI-TOF MS approach for analyzing nasal swabs from patients with suspected COVID-19 [76].

The authors evaluated the performance of the MALDI-TOF MS method using SARS-CoV-2 RNA PCR positive and negative samples in order to determine the accuracy, the positive percent agreement (PPA), and the negative percent agreement (NPA) of the MALDI-TOF MS method as compared to the PCR method. They collected 226 nasal swab samples that were analyzed both by RT-PCR and by MALDI-TOF MS. Normalized mass spectra were subjected to ML analysis by the Auto-ML MILO (Machine Intelligence Learning Optimizer) platform [96]. The MILO as ML platform consists in a series of algorithms, scalers, scorers, and feature selectors/transformers, which are used to generate models that are then statistically assessed to ultimately identify the best performing model for a specific purpose. Briefly, its infrastructure consists of two datasets: the balanced dataset used for training and validation, and an unbalanced dataset for generalization/secondary testing. Finally, the selected model is deployed and used to test new data and make predictions. Among the 226 nasal swab samples, 27 were eliminated due to polymer contamination of the sample. Overall, 199 samples were analyzed by both MALDI-TOF MS and RT-PCR; out of these, 107 samples were COVID-19 positive (28 asymptomatic and 79 symptomatic) while 92 were COVID-19 negative (Table 1). The data related to MALDI-TOF spectra obtained from these 199 samples were divided into Datasets A and B, with dataset A used as a training and initial validation dataset (which included 42 COVID-19 negative cases and 40 COVID-19 positive ones). Optimized models obtained from Dataset A were then tested with dataset B, used as generalization test set to assess their true performance (constituted by 50 negative cases and 67 positive cases).

MILO produced a total of 379,269 models, two of which—DNN and GBM—showed high performance characteristics (Table 1). The authors described their ML-enhanced MALDI-TOF approach as an attempt to address both throughput and speed limitations observed in molecular platforms; interestingly, the authors demonstrated the ability of both PCA and ML models to classify MALDI-TOF mass spectra for discriminating positive and negative COVID-19 cases.

The high-throughput performance of this platform was also demonstrated by the short time required for the analysis (total turnaround time was less than 1 h, with the potential of performing up to 1104 analyses per day, per instrument) as compared to commercial RT-PCR platforms, which need, for example, batch testing for optimal reagent use.

Another investigation that described the analysis of NP samples by MALDI-TOF MS coupled with ML was performed by Deulofeu and colleagues [77]. In particular, they used different ML approaches for the analysis of the MALDI-TOF mass spectra from 237 NP samples in order to identify a signature characterizing negative and positive samples for SARS-CoV-2 infection (Table 1). These samples were analyzed by both RT-PCR and MALDI-TOF MS. The mass spectra obtained were used to build different ML models in order to identify the best model that would be able to distinguish the positive COVID-19 cases from negative ones. All the parameters of a MALDI-TOF MS approach, including the matrix, sample dilution, and mass range, were optimized. Interestingly, the effect of the use of different viral transport media on the end results was also investigated. MS data analyzed by the ML models were able to identify the more appropriate viral transport media for the detection of SARS-CoV-2 infected samples. In particular, the best results were achieved using the SVM model, which obtained elevated levels of accuracy, sensitivity, and specificity (Table 1). These data demonstrated the utility of the developed method for the detection of SARS-CoV-2-positive samples and the simplicity, safety, rapidity, and cheapness of MALDI-TOF MS as a diagnostic test. The authors estimated the cost of MALDI-TOF MS to be 25% more than the cost of the RT-PCR analyses. They also stated that the time required to receive results is around 6 h for a conventional RT-PCR, whereas the analyses of the NP samples by MALDI-TOF MS take less than one-third of this (Table 1).

Finally, Rocca and colleagues also investigated the potential role of MALDI-TOF MS combined with ML methods in the identification and the discrimination between COVID-19 positive samples and COVID-19 negative ones [78].

They used NP swab samples from 311 patients that were analyzed by MALDI-TOF MS analysis without processing (Table 1). They used 20 main spectrum profiles obtained from nine COVID-19 positive samples, eight COVID-19 negative samples, and three positive samples for other respiratory viruses to create a new "in-house" database (named BE COVID-19). Using this database, they searched for potential discriminatory peaks that could differentiate positive from negative samples, using two different software (Flex analysis v3.4 and ClinPro tools, Bruker Daltonik GmbH, Bremen, Germany). From the evaluation of parameters and statistical analysis, six peaks at m/z 3372, 3442, 3465, 3488, 6347, and 10,836 demonstrated a decrease or an absence of COVID-19 positive samples and were considered as potential biomarkers (Table 3).

The authors applied ML algorithms and used 432 spectra (obtained from 55 COVID-19 positive samples, 57 COVID-19 negative samples, 24 samples positive for the influenza virus, and 8 other respiratory virus samples) as a training set for the generation of three classification models; to analyze the performance of this approach based on ML algorithms with the combination of potential biomarkers, a validation test was performed on 501 spectra (167 clinical samples), obtaining an accuracy of 67.66%, a sensitivity of 61.76%, and a specificity of 71.72%.

These preliminary results demonstrated that the method still needs to be improved, and the authors stated that further studies on a larger cohort of patients, evaluating different extraction methods and improving ML algorithms, will be performed. An important weakness of this study might be the limited number of samples used to build the "in-house" database. In fact, a larger number of clinical samples are required to obtain a reliable spectrum profile library, in order to reduce the inter-individual variability effects and to determine specific discriminatory peaks with more confidence.

Yan et al. utilized a high-throughput serum peptidome profiling method based on MALDI-TOF MS for the detection of COVID-19 [79]. They analyzed serum samples from a total of 298 individuals: 146 COVID-19 patients (classified into mild, typical, severe, and critical) and 152 control patients (including 73 non-COVID-19 patients with similar clinical symptoms such as fever/cough, 33 tuberculosis patients, and 46 healthy individuals) (Table 1).

After sterilization, the serum samples were profiled by MALDI-TOF MS. The MALDI-TOF data of COVID-19 patients and control samples were randomly split into training

cohorts, with 198 samples (101 controls and 97 patients), and validation cohorts, with 100 samples (51 controls and 49 patients). Using ML methods, twenty-five peaks were identified as the distinctive features between COVID-19 patients and control participants, with statistically significant differences (Table 3). Most of the peaks were located in the range of m/z 5000–30,000. Distinctive features were observed: e.g., m/z = 6357, 6654, 6639, 13,886, and 28,232 were significantly down-regulated in the COVID-19 group, while m/z = 7614, 15,123, 15,867, and 28,091 were significantly up-regulated in the COVID-19 group (Table 3). The classification model for the detection of COVID 19 was constructed with these 25 feature peaks in the training cohort by using eight ML methods.

The ROC curves and AUC were evaluated for each of the ML models. Later, the efficiency of these models was assessed in the validation cohort, which was independent from the samples included in the training cohort. The Principal Component Analysis (PCA) of the 25 features demonstrated that the COVID-19 patients and control cases in the test group were well-separated. Among all the models, the LR model demonstrated the best classification performance and it was considered the most recommended by the authors for future applications in the detection of COVID-19, with the highest accuracy (99%), sensitivity (98%), precision (100%), and specificity (100%) (Table 1).

Starting from the observation that the symptoms of SARS-CoV-2 infected patients are frequently shared by patients affected by other respiratory infections, the authors highlighted that this could lead to the misinterpretation of results; so they emphasized the ability of this platform to discriminate COVID-19 patients from non-COVID-19 subjects with similar clinical symptoms. The authors concluded that these results also demonstrated that MALDI-TOF-based serum profiling could be a powerful tool for screening, routine surveillance, and diagnosis of COVID-19 in populations. However, it is important to note that the sampling time of the COVID-19 serum samples analyzed ranged from 3 to 28 days from the onset of symptoms, which consists in a relatively long period of disease progression. This condition could represent one potential limitation of this study, as a screening method should be able to detect the virus infection as early as possible in order to avoid an uncontrolled viral spread and to stop the pandemic.

5. Discussion

5.1. Comparison of MALDI-MS vs. RT-PCR Techniques for SARS-CoV-2 Detection: Advantages and Limitations

As a resource for new, alternative and/or complementary, rapid COVID-19 diagnostic tests, this review explored the recent developments and applications of MALDI MS-based technologies for the accurate and unambiguous SARS-CoV-2 clinical diagnosis, which may help to control the COVID-19 pandemic.

From the perspective of a high-throughput, routine, diagnostic clinical setting, a viral infection diagnostic tool should consist of an integrated analytical platform, which needs to be fast, sensitive, highly specific, reproducible, and cost-effective. MS techniques have rapidly complemented or replaced conventional methods in clinical diagnostic laboratories and have been successfully implemented in the investigation of viruses and their pathogenesis [17,21–27,97–99]. In particular, MALDI-MS has become an indispensable and versatile method for biochemical and clinical investigations [100,101]. MALDI-MS represents one such technology, characterized by its ease of use, high-throughput capabilities, and cost effectiveness, thus becoming a routine tool in clinical microbiology laboratories and also proving to be capable of supporting clinical decision-making [36,64].

However, unlike what happens with bacteria, neither MS nor MALDI-MS currently have a low impact on virus detection in the routine diagnostics of clinical samples [102]. In fact, the approach used for biotyping is not well-suited for the identification of viruses for some reasons. First of all, viruses cannot be isolated by simple methods, unlike bacteria. As a matter of fact, bacteria are routinely grown and isolated from colonies, while viruses can be found in a complex cellular background, making their detection more difficult. Hence, the sensitivity is severely limited by the dynamic range of the MS instrument used. A second reason is that bacteria contain significantly more proteins than viruses, with poor

signals in the m/z below 12,000, the range covered by whole-cell MALDI-TOF MS. Hence, the interpretation of low-resolution mass spectra made up of few viral peaks in a complex human background is more challenging.

As a matter of choice, the clinical sensitivity and specificity of each diagnostic tool should be evaluated in various clinically relevant real-life situations (for example, the viral load, the site and the quality of sample collection, timing, and illness severity). The current gold standard technique for the molecular diagnosis of SARS-CoV-2 infection is the RT-qPCR, which allows for the analysis of thousands of samples in a single day and shows a high testing sensitivity of 95% [103] and a low limit of detection of <10 copies/reaction [104] under ideal circumstances. Nevertheless, several investigations have reported that clinically evident COVID-19 infections often go undetected by SARS-CoV-2 RT-PCR testing [10,105–108]; the evidence that virus shedding can occur at undetectable levels during the very early and late stages of SARS-CoV-2 infection demonstrates that RT-PCR results should always be interpreted in a wider context. Indeed, the most efficient strategy for the diagnosis of SARS-CoV-2 infection in suspected patients should encompass a combination of SARS-CoV-2 detection by RT-PCR with clinical and epidemiologic observations (symptoms, previous exposure to virus, negative diagnostic tests for other respiratory diseases), whereas additional follow-up testing with RT-PCR should be required from patients with initially negative results and high suspicion of COVID-19.

The time required for conventional PCR can vary from about 4–6 h for sample processing (including sample preparation, RNA extraction, reverse transcription PCR, and readout of amplified DNA products), up to a few days, considering the time needed for the eventual transport of the specimen to the laboratory.

Most of the reports described here that are related to Biotyping, Proteotyping, and Genotyping approaches are proof-of-principle studies showing the feasibility of viral protein or viral nucleic acid detection by MALDI-MS, which often do not involve stress testing in low viral load samples. Nevertheless, as reported in Tables 1 and 2, the specificity, sensitivity, and the detection limit of the SARS-CoV-2 virus by MALDI-MS diagnostic methods were high and comparable to reference methods such as PCR.

The MALDI-MS detection of a virus has a fast turnaround time from sample collection to diagnosis (Table 1). The time required is mainly encumbered by the procedures needed for sample processing, considering that spectral acquisition could be performed in an automated or semiautomated manner.

Furthermore, MALDI-MS analysis is cost-effective despite the initial investment in expensive equipment. In fact, especially in the case of ultra-high-resolution MS, the purchase of the mass spectrometer instrument accounts for the major cost; however, it is a one-time cost and not inconsistent with the costs required for multiple PCR sequencers and associated specialized reagents and equipment [74].

5.2. Pre-Analytical and Analytical Issues for Molecular Detection of SARS-CoV-2

As a rule, the reliability of each diagnostic tool is influenced by several factors which could reduce the sensitivity and specificity of the obtained results, and which have to be estimated to avoid erroneous interpretations by the clinical laboratories [16,109]. There are several critical issues and challenges affecting the diagnosis of SARS-CoV-2 infections, including sampling operations, specimen source, and sampling time, which could have an impact on the end result. It is essential to handle bodily fluids according to standardized procedures in order to distinguish reliable molecular biomarkers and to assess the influence of pre-analytical parameters on the final result in order to avoid artifacts. As revealed by the literature reviewed in the previous sections, there is high variability in all of the abovementioned critical technical factors; for example, most studies do not clarify if the tested samples were analyzed by MALDI-MS immediately after collection or if they had been deep-frozen until analysis. This represents an important limitation, considering that several pre-analytical and analytical variables, including storage time and temperature, could alter the analysis of the MS profile of biological specimens, and that several studies

demonstrated that the proteins could undergo degradation processes [51,110–113]. Thus, especially for the biomolecular host profiling studies, it is not possible to exclude the fact that the observed differences in the molecular fingerprints obtained by MALDI-MS could derive from the degradation of the molecular species due to the action of endo-proteases, including those of microbial origin in the nasal environment, or from the inherent biological variability among subjects, also due to genetic factors affecting the phenotype. Worthy of note is the fact that most studies do not clarify if the tested samples were collected at the time of symptom onset rather than time of exposure, disregarding another important issue which could lead to high variance in the detection of SARS-CoV-2 infection in the first few days after virus exposure. The success of an accurate diagnostic procedure mainly depends on the collection of proper specimens at the right time of infection, and from the appropriate anatomic location/organ.

5.3. Detection of SARS-CoV-2 in Different Types of Clinical Specimens

Respiratory sampling is the preferred and widely approved method for SARS-CoV-2 detection by RT-qPCR. The choice of optimal clinical specimens takes into consideration several factors such as ease of accessibility, non-invasive collection procedure, a lower risk to health care professionals during sample collection, and good viral loads for higher possibilities of detection. NP and/or OP swabs are best recommended for the screening or diagnosis of an early infection, when low viral loads are known to occur and false-negative results can derive from differences in analytical sensitivity among methods [16,114]. For these reasons, most of the studies reviewed here are based on the use of NP or OP samples [71,72,74–78]. Interestingly, nasal swab samples were directly applied to MALDI-MS, and spectral readouts were used to identify infected or non-infected subjects; consequently, non-purified viral samples were used and any virus would be markedly diluted [71,72,74–78]. These conditions have allowed for a higher viral load that not only facilitates the detection by MS but also reduces the time needed for the experimental procedure and analysis. On the other hand, NP or OP swabs show limitations in sample collection and present a risk to medical staff through sneezing or coughing, which can lead to aerosol exposure of viral particles [115].

An alternative option for collecting upper respiratory tract specimens in order to detect SARS-CoV-2 is self-collected saliva or gargle specimen [93]. Saliva is a reliable biological fluid that can easily be provided by the patient; it does not require healthcare personnel in a screening program [93,116]. In fact, a mass screening program should rely on devices that can be used by non-medical staff to promptly assess whether an individual is positive to COVID-19 [117]. Moreover, a gargle sample is obtained from an easy-to-perform procedure and is comfortable for the patient. Reliable and rapid diagnostic techniques based on the use of saliva or gargle specimens might represent a cost-saving procedure considering that their collection does not require the need to develop and maintain specific infrastructure for swab collection as well as the need for dedicated healthcare personnel. The studies reviewed here, which reported the use of saliva/gargle specimens, analyzed the feasibility of these biological fluids as a diagnostic sample for detecting SARS-CoV-2 infection [69,70,73]. It is worth noting that compared to swab-based diagnostics, several studies majorly revealed a higher viral load in NP than in saliva specimens [118], probably due to dilution effects. However, it should be noted that the possibilities to detect SARS-CoV-2 infection from saliva, gargle, NP, or OP specimens could decrease with the time since the onset of symptoms, leading to a higher risk of false-negative results [16]. In fact, in later infection, the main site of replication could shift to the low respiratory tract, and repeated testing or lower respiratory tract specimens may represent the alternative choice [115,119].

Serum samples could be used to understand the molecular mechanism for the diagnosis of COVID-19. As a consequence of viral infection, serum may contain mediators produced by the systemic effects or released to the lung, and this could reflect the physiological or pathological state [30].

Serum samples, and more generally, serological methods, could play a key role in the epidemiology of COVID-19 and in defining the immune status of asymptomatic patients, but they are not recommended for the screening or diagnosis of early infections [120]. However, serology could contribute to the confirmation of the diagnosis of COVID-19 by providing information about the type and the concentration of various immunoglobulins generated after SARS-CoV-2 infection [121].

5.4. Asymptomatic Infection and the Spread of New SARS-CoV-2 Variants

Another critical issue is the limited resources employed to facilitate and enable the widespread rapid screening of asymptomatic individuals necessary to control the potential of silent chains of virus transmission. Asymptomatic patients are the carriers of the live, replicating virus and contribute to the unknown viral transmission and spread. In some cases, the methods used for SARS-CoV-2 detection in these subjects can be less sensitive than in the symptomatic ones [109,122,123]. It is worth noting that only a limited number of studies reviewed here have considered the asymptomatic population [70,76]. However, they demonstrated that the MALDI-TOF MS platform was able to detect SARS-CoV-2 infection in asymptomatic individuals, and with elevated accuracy [76] (see Table 1), which could be of paramount importance for the reopening of businesses, recreational facilities, schools, and all non-hospital settings.

In addition to asymptomatic infection, SARS-CoV-2 variants are another challenge for virus detection [123,124]. In fact, new variants caused by mutations in the genome of the SARS-CoV-2 are now emerging as the virus spreads throughout the world, and this is of great relevance considering that such mutations may alter various aspects of virus biology such as pathogenicity, infectivity, transmissibility, and/or antigenicity [125]. Nowadays, the mutations and evolution of SARS-CoV-2 are mainly traced by next generation sequencing, but routine genomic testing is expensive and difficult to perform in real time due to the lack of resources and expertise in many areas [126,127]. Additionally, emerging variants raise the risk of target dropouts and false-negative results due to primer/probe binding site mismatches. Therefore, alternative and/or complementary methods of rapidly detecting and monitoring the evolution of virus strains are of vital importance.

Very recently, some studies employed MALDI-MS to study and distinguish strains of the major variants of concern using mass signatures [124,128,129]. These studies also demonstrate how such MALDI-MS data can be used to chart viral evolution and to construct mass-based phylogenetic trees, similar to those obtained using conventional gene sequence phylogenetics. The use of MS datasets to track the evolution of SARS-CoV-2 in order to identify such mutations that may limit detection by PCR or those that allow immune escape or reduce the efficacy of existing vaccines and/or therapies have great future potential.

6. Conclusions

Laboratory SARS-CoV-2 testing assays are the primary mitigation measure for the prompt identification and isolation of infected individuals. In fact, the rapid and accurate detection of SARS-CoV-2 infection and the close monitoring of persons in contact with cases are key steps in managing the COVID-19 pandemic, until vaccines can be extensively administered or until antiviral drugs become widely available. In this context, MS-based technologies may represent an alternative resource or may support PCR-based methods as a diagnostic test in the fight against the novel coronavirus. To date, MALDI-MS is routinely used in clinical practice for the rapid, unambiguous, and cost-effective identification of pathogens in biological samples. It is worth noting that several MS-laboratories around the world have been working to deliver guidelines and protocols on sample collection, processing, and data analysis in order to develop rapid and robust methods for the high-throughput screening of SARS-CoV-2 infection. Furthermore, clinical trials using MALDI-MS are currently ongoing for the management of the COVID-19 pandemic [130,131]. In particular, one of these studies is based on MALDI-TOF MS profiling and combined

with ML methods to detect individuals infected with SARS-CoV-2 from saliva samples, while the other one is based on the MALDI-TOF MS analysis of both saliva and blood samples to search for discriminating profiles between COVID-19 and non-COVID-19 individuals [130,131]. Nevertheless, some limitations still exist for the applications of MALDI-MS technology in routine virus diagnostics. In fact, unlike what happens for bacteria, in the case of biotyping approaches, the major limitations could arise from the relatively low protein content of viruses, the higher molecular weight of viral proteins (>20,000 Da), and a probable carryover of debris from the cell substrate in which the viruses are cultured in vitro.

On the other hand, despite the high costs of the instrument, the high-resolution MALDI MS with proteotyping strategies, based on SARS-CoV-2 signature peptides, has demonstrated excellent performance characteristics especially for the high-accuracy detection of SARS-CoV-2. Considering high accuracy, high throughput, and speed of analysis, the use of more innovative MS-based tools and assays represents an essential and feasible complement to PCR.

MALDI-MS-based genotyping approaches also enable the high-throughput and low-cost detection of SARS-CoV-2. Nevertheless, the level of accuracy of these approaches is not yet comparable to those of proteotyping strategies. In any case, conventional tests such as PCR or immunoassay have become insufficient, also considering the shortages of medium, reagents, collection devices, and consumables that hinder day-to-day laboratory operations and the ability to improve testing capacity. In this scenario, MALDI-MS-based genotyping strategies could represent reliable diagnostic techniques that require alternative consumables. Moreover, recent investigations have demonstrated the ability of these platforms to assess unfolding genomic variation in a timely manner, and have highlighted the potential of diagnostic results to serve as a reliable system for the detection of emergent SARS-CoV-2 variants of concern [128,129].

Finally, regarding the proof-of-principle studies, the biomolecular host profiling approaches that assess host response to predict infection are a further intriguing alternative to the current testing methods for the identification of SARS-CoV-2 infected individuals. However, viral load differs among infected individuals, in particular between symptomatic and asymptomatic subjects, which leads to a broad variation in host response. Therefore, these approaches warrant further investigations that would test their threshold for the detection of viral infection and their ability to differentiate types of infectious or non-infectious causes of host modulation, in order to avoid confounding results. Additionally, these strategies also have multiple desirable features for a clinical test during the COVID-19 pandemic, which includes minimal sample preparation, few reagents, and rapid and high-throughput data acquisition.

Arguably, as part of a pandemic preparedness plan, a massive amount of novel scientific knowledge as well as the use of innovative MS-based tools and assays will support the development of diagnostics facilitating a reliable response to emerging infectious diseases.

Certainly, the expanded application of MS-based technologies and related multi-omics strategies could become one of the reference methodologies for other future epidemics/pandemics that may arise.

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