Research Article

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Digital methylation-specific PCR: New applications for liquid biopsy

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Abstract: Epigenetic analysis is a fundamental part of understanding pathophysiological processes with potential applications in diagnosis, prognosis, and assessment of disease susceptibility. Epigenetic changes have been widely studied in chronic obstructive pulmonary disease (COPD), but currently, there is no molecular marker used to improve the treatment of patients. Furthermore, this progressive disease is a risk factor for the development of more severe COVID-19. Methylation-specific polymerase chain reaction (MSP-PCR) plays an important role in the analysis of DNA methylation profiles, and it is one of the most widely used techniques. In this context, the combination of MSP-PCR with emerging PCR technologies, such as digital PCR (dPCR), results in more accurate analyses of the DNA methylation profile of the genes under study. In this study, we propose the application of the MSP-dPCR technique to evaluate the methylation profile of the *ADAM33* gene from saliva samples and lung tissue biopsies of patients with COPD and COVID-19. MSP-dPCR generated a measurable prediction of gene methylation rate, with the potential application of this combined technology for diagnostic and prognostic purposes. It has also proven to be a powerful tool for liquid biopsy applications.

Keywords: DNA methylation, polymerase chain reaction, epigenetic, COVID-19, chronic obstructive pulmonary disease

Introduction

Studies of the epigenetic mechanisms of DNA modification reveal information about the gene expression profile that is directly linked to the phenotype of the organism [1]. DNA methylation events have been extensively studied and associated with several biological processes, including aging, pathologies, or susceptibility to such conditions [2–4]. DNA methylation occurs at cytosines flanked by guanines, the so-called CpG islands. Methylation of these islands has been implicated as a mechanism that prevents gene transcription. Specifically studying the methylation of a gene or its promoter can provide insights into the gene's activation or inactivation across various contexts [1,5]. To access DNA methylation, various techniques are used to differentiate methylated or unmethylated cytosines by treating DNA with sodium bisulfite [6]. These techniques include DNA sequencing, microarrays, and for specific regions, methylation-specific polymerase chain reaction (MSP-PCR) [7]. The MSP-PCR technique was first described by Herman et al. (1996) [8] and predicts that sodium bisulfite-treated DNA will amplify under two conditions: either as methylated (M) or unmethylated (U) sequence. This determination of methylation status occurs after DNA sequencing determination of methylation status in specific samples [9,10]. To achieve this, primers are designed to amplify regions rich in CpG dinucleotides (CpG islands) and contain as many downstream CpGs as possible in the 3′ region of the sequence. The two primer pairs are designed to amplify exactly the same region. The PCR products from this amplification are visualized by electrophoretic separation, making it possible to

Equality contribution.

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identify the products of the unmethylated, methylated, or both conditions [8,11]. More recently, this has been done through quantitative PCR (qPCR). Although simple, inexpensive, and highly sensitive, the classical MSP-PCR technique [8] is not quantitative and does not allow the measurement of methylation levels on DNA samples [12,13].

In recent years, PCR techniques have been improved and expanded with the development of various platforms for qPCR and, more recently, for digital PCR (dPCR) [14]. dPCR provides researchers with an attractive solution for absolute DNA quantification to aid in detecting genetic variants, including those found in liquid biopsies, where it demonstrates high sensitivity [15–17]. Copy number variation analysis or gene expression studies are also common applications of this technology.

dPCR is based on the individualization of PCR into multiple partitions, which function as independent reactions through Poisson's random distribution. It allows absolute quantification of the original copy number of the amplified gene without interference from parameters such as amplification efficiency, by endpoint detection of amplified products and the binarization signal: (0) absence or (1) presence [18,19]. The dPCR technique includes high sensitivity and specificity analysis for low-copy number genes, following the standards established by the Digital PCR MiQE guideline (2020) [18]. The high detection sensitivity of this method has led to increased awareness and use of this technique for diagnostic purposes, particularly in the context of liquid biopsies [16,20]. Liquid biopsies have gained considerable attention over the past decade as an attractive method for noninvasively detecting, screening, and monitoring disease in various health conditions. Blood, body fluids such as urine or stool, saliva, sputum, or various secretions offer numerous advantages over traditional biopsies, such as comprehensive tissue sampling, repeatable sample collection during the disease's progression, and accessibility in vulnerable individuals [21–23]. However, a crucial question in personalized medicine revolves around determining the most suitable biomarkers for health monitoring.

A biomarker is defined as a measurable, objective indicator of the normal and abnormal physiological state of an individual, indicating any change in that state. If these biomarkers are properly applied and patients are properly educated on how to respond to altered biomarker levels, this could immensely benefit rapid and personalized healthcare [24]. Disease detection by liquid biopsy is generally achieved by detecting molecular markers of specific genetic or epigenetic changes in tissues throughout the body [25]. Accordingly, the use of saliva in the study of respiratory diseases could assist in pathology monitoring. Saliva is a non-invasive biofluid that is easy to collect, transport, and store [26]. Because of its accessibility and association with systemic diseases, saliva stands as a prime candidate for easy health status monitoring [24,27]. The term asthma–COPD overlap (ACO) has been used to identify a heterogeneous condition where patients exhibit airflow limitation that is not entirely reversible, along with clinical and inflammatory characteristics present in both asthma and COPD. However, the diagnosis of ACO can be difficult in clinical practice, since there is still controversy regarding its definition, pathophysiology, and impact [28]. The discovery of molecular markers might redefine the boundaries of COPD diagnosis in clinical settings. Furthermore, COPD was often associated with COVID-19 severity during the pandemic year, due to the high mortality rate within this patient population [29,30]. As epigenetic factors influence both diseases, it is important to define how the group could be better diagnosed or need a different treatment, for example.

The ADAM33 protein, part of the ADAM protein family (A Disintegrin And Metalloproteinase), is encoded by the ADAM33 gene located on chromosome 20p13 and plays a vital biological role by activating Th2 cytokines and growth factors [31]. Soluble ADAM33 protein can increase angiogenesis, impacting lung functions and causing airflow obstruction independent of inflammation [32]. As ADAM33 expression is restricted to mesenchymal cells, including airway fibroblasts, myofibroblasts, and smooth muscle cells [33], it is proposed that dysregulated ADAM33 activity is associated with airway remodeling and bronchial hyperresponsiveness, considered a risk factor for asthma [32,34], due to increased expression in this disease [34]. In the context of COPD, it is well established that ADAM33 is involved in the pathophysiology and inflammatory response, including alterations in pro- and pre-inflammatory cytokines [35–37]. Several studies investigate the role of soluble ADAM33 and the upregulation of its mRNA, crucial in extracellular matrix damage and as part of the pathogenic mechanism of COPD [38,39].

It is known in the literature that the ADAM33 gene has well-defined epigenetic mechanisms in other non-communicable diseases, such as in breast cancer, where ADAM33 is regulated by DNA methylation, and its differential expression can influence the disease severity [40]. Although there is no scientific evidence showing the epigenetic profile of this gene in patients affected by COVID-19, a recent study found that SARS-CoV-2 infection, evolving into severe COVID-19, was associated with DNA methylation changes throughout the genome of peripheral blood mononuclear cells. When comparing severe COVID-19 with uninfected controls, 40,904 differentially methylated loci were identified [41]. Therefore, ADAM33 could be an excellent candidate as both a biomarker and a treatment target for COPD.

The selection of validated biomarkers, combined with high-quality detection tools like dPCR, could make saliva an important biopsy fluid for the detection of respiratory diseases. Combining this knowledge, this study aims to adapt the MSP-PCR protocol developed by Herman et al. [8] to the dPCR platform (MSP-dPCR) using QIAGEN technology. The objective is to employ this technique to assess the methylation profile of the promoter region of the *ADAM33* gene in saliva samples from patients with chronic obstructive pulmonary disease (COPD), both with and without COVID-19. This highly sensitive and specific MSP-dPCR bisulfite conversion assay promises to be an essential tool for further understanding the mechanistic role of DNA methylation of genes that may lead to human disease, providing broad population-based results through an affordable platform capable of detecting specific DNA methylation patterns.

Materials and methods

Saliva and lung samples

Saliva samples (n = 20) were collected between February and April 2022 from COPD patients, with or without COVID-19 diagnosed by PCR for the SARS-CoV-2 virus. Patients were seen at the COPD outpatient clinic and/or the post-COVID outpatient clinic of the Pulmonology Department of the Clinical Hospital Complex – UFPR/EBSERH – Curitiba, Paraná, Brazil. During the appointment, patients collected approximately 1 mL of raw saliva in sterile 2 mL tubes, which were then stored at −20°C until further processing. For the COVID-19 positive and negative controls, samples were obtained from the Laboratory of Human Cytogenetics and Oncogenetics (LabCHO) during the screening of COVID-19-contaminated individuals performed by the Department of Biological Sciences of the Federal University of Paraná. Lung samples (n = 29) were collected from patients who died due to COVID-19, while they were bedridden and connected to mechanical ventilation systems. The collections were performed by surgical biopsy by the medical team from the Pontifical Catholic University of Paraná at a tertiary hospital in Curitiba, Paraná, Brazil. The collections were conducted between June and August 2020 and March and August 2021. All samples were stored in an ultra-freezer at −80°C until processing.

DNA isolation and sodium bisulfite treatment

To obtain DNA from saliva samples, the entire 1 mL of saliva underwent the QIAamp Circulating Nucleic Acid protocol (Qiagen, Hilden, Germany) following the manufacturer's specifications. To obtain DNA from lung samples, approximately 1 mm^3 of tissue was transferred to 2 mL tubes containing 1 mL of TES buffer (Tris-HCl, pH 8.0, at 10 mM, EDTA at 50 mM, SDS at 0.5%, RNase at 0.1 mg/mL, and Proteinase K at 0.05 mg/mL). The samples were homogenized in a TissueLyser LT device (Qiagen, Hilden, Germany) at 40 Hz cycles for 40 s and processed according to the specified protocol [42]. The purified DNA was quantified using a NanoDropLite spectrophotometer (ThermoFisher Scientific, Waltman, MA, USA) and subjected to agarose gel electrophoresis (0.8%). Approximately 2 µg of purified DNA was treated with sodium bisulfite using the Epitec Plus Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Primer design

The primers were selected according to established methods in the referenced study [40] and were presumably chosen for their specificity and effectiveness in amplifying the target regions of interest in these genes. The primer sequences are described in Table 1.

MSP-dPCR

Approximately 50 ng bisulfite-treated DNA was added to a 40 µL reaction containing 0.4 µM oligos and 1× EvaGreen (Qiagen, Hilden, Germany). Methylated (M) and unmethylated (U) reactions were performed simultaneously under the same conditions and a master mix was prepared separately using the specific primers for the U/M conditions. Since COVID-19-positive and -negative samples were used

Table 1: Sequence of oligos used as primers for the MSP-dPCR

Gene	Forward 5′–3′	Reverse 5′–3′
ADAM33 M	GTTTGAGGTTGTAT**CG**GGTA	ACT**CG**CAACTC**CG**ACTC**CG**
ADAM33 U	GTTTGAGGTTGTAT**TG**GGTA	ACT**CA**CAACTC**CA**ACTC**CA**

M, methylated; U, unmethylated.

as controls, no 100% methylated or 100% unmethylated controls were needed for this assay. The QIAcuity 24-well nanoplates with 26,400 partitions were used on the QIAcuity 5 plex Digital PCR System (Qiagen, Hilden, Germany), where the nucleic acid template was randomly distributed across all available partitions and individually amplified. PCR cycling conditions for *ADAM33* are as follows: 95°C for 2 min; 40 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 20 s, and extension at 72°C for 15 s, followed by 5 min at 40°C. The amplification target was detected at the end of the PCR process by assessing the presence or absence of fluorescence resulting from intercalating dyes. Since the PCR is randomly partitioned, it is possible for a positive reaction to contain more than one target molecule, so the Poisson model is utilized to determine the probability of a microreaction receiving zero, one, two, three, four, or five copies of the target molecule. The total number of copies of the target molecule in all valid partitions of a well was calculated by multiplying the average number of target molecules per partition by the number of valid partitions. Copies per microliter can also be calculated based on the known number of target copies per partition and the partition volume. All analyses were performed using the QIAcuity Suite software (Qiagen, Hilden, Germany).

Statistical analysis

The absolute quantification data (copies/µL) obtained from MSP-dPCR were analyzed using the Student's *T*-test. A *P*-value of <0.05 was considered statistically significant.

The data for unmethylated *ADAM33* and methylated *ADAM33* from the same DNA input concentration were processed using the formula below [43]. The resulting values are expressed as percentages of methylated and unmethylated copies.

$$\text{Percentage of methylation} = \left[\frac{M}{(M+U)}\right] \times 100,$$

$$\text{Percentage of unmethylation} = \left[\frac{U}{(M+U)}\right] \times 100,$$

M is the methylated copies/µL and U is the unmethylated copies/µL.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee. This research has been approved by the Ethics and Research Committees of CONEP (National Commission for Ethics in Research) under the protocol codes CAAE 30188020.7.1001 and 31687620.2.0000.0096.

Informed consent: Informed consent has been obtained from all individuals included in this study.

Results

The saliva and lung samples were initially categorized into three groups to investigate the range of hypothesized molecular markers. The first group comprised patients without COVID-19 or COPD (control group). The second group included patients who tested positive for SARS-CoV-2 via qPCR at the time of sample collection (COVID-19-positive group). The third group encompassed saliva and lung samples from COPD patients diagnosed with COVID-19 by qPCR at any time between 2021 and 2022 (COPD + COVID-19 group). Additionally, a fourth group of patients diagnosed solely with COPD was included in the saliva analysis. The samples then underwent the procedure outlined in the flowchart below (Figure 1).

After DNA extraction and sodium bisulfite treatment, all the samples within the same group were diluted to a similar concentration. For saliva samples, the range was between 44.6 and 69.2 ng/µL (median = 58.9 ng/µL; Q_1 = 53.95 ng/µL; Q_3 = 62.95 ng/µL) in the COPD + COVID-19 group. The COVID-19-positive control had a concentration

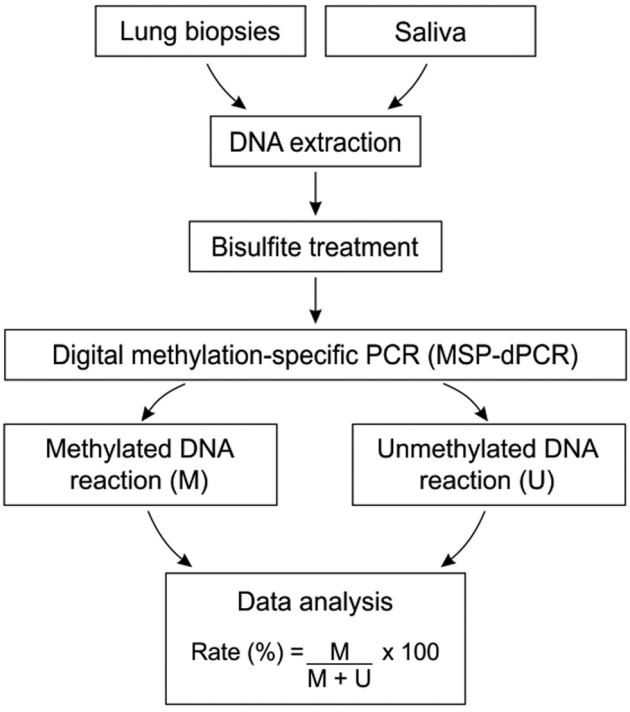

Figure 1: Flowchart of the methodology used to evaluate DNA methylation of the ADAM33 gene. M, methylated copies; U, unmethylated copies.

Figure 2: Fluorescence intensity of the analyzed partitions in (a) methylated and (b) unmethylated ADAM33 assays in saliva samples. The red line indicates the threshold between positive and negative partitions (gray dots). *dot plot obtained from the QIAcuity 5 plex (QIAGEN) equipment showing the rainfall on the samples analyzed.

of 30.1 ng/μL, while the COVID-19-negative control group had 24 ng/μL. In the case of lung samples, the range was from 228.5 to 229.8 ng/μL in the COPD + COVID-19 group. The concentration of the COVID-19 group ranged from 63.0 to 256 ng/μL (median = 156.75 ng/μL). Solely within the COPD group, the range was between 11 and 47.8 ng/μL (median = 12.8 ng/μL; Q_1 = 11.75 ng/μL; Q_3 = 17.45 ng/μL). After sample standardization by the guidelines (dMIQE, Table Supplementary material), MSP-dPCR was performed for the *ADAM33* gene [20].

In saliva samples, the range of *ADAM33* methylated copies was between 19.25 and 53.08 copies/μL in the COVID-19 group. In the COVID-19 + COPD group, the range of *ADAM33* methylated copies was between 16.29 and 632.8 copies/μL. In the negative control group, the range of methylated copies was between 15.32 and 329.1 copies/μL. The *ADAM33* unmethylated copies in the COVID-19 group ranged from 25.64 to 31.48 copies/μL. In the COVID-19 + COPD group, the range was from 7.721 to 138.9 copies/μL. In the COPD group, the range was from 15.97 to 605.8 copies/μL. In the negative control group, the unmethylated copies ranged from 16.57 to 31.61 copies/μL (Figure 2). We observed a higher number of methylated copies by μL of DNA in the control group and a higher number of unmethylated copies in the COPD group ($p < 0.005$). For the COPD + COVID-19 group, we observed a significant number of unmethylated copies by μL of DNA. In our analysis by copies/ng, we found a significant value to the unmethylated copies in the COPD group (Figure S1). The confidence interval of MSP-dPCR data for ADAM33 in saliva samples is presented in Table 2.

For lung samples, the COVID-19 group showed a range of methylated *ADAM33* copies between 0.209 and 0.532 copies/μL, while the range of unmethylated copies was 0.16–350 copies/μL. In the COVID-19 + COPD group, the range of methylated *ADAM33* copies was between 1.30 and 1.84 copies/μL, with unmethylated copies ranging from 6.11 to 7.90 copies/μL (Figure 3). We observed an increase in unmethylated

Table 2: Confidence interval of MSP-dPCR data for ADAM33 in saliva samples

	Control U	Control M	COVID-19 U	COVID-19 M	COPD + COVID-19 U	COPD + COVID-19 M	COPD U	COPD M
Lower 95% CI of mean	−361.4	−333.5	−79.19	−73.53	24.32	31.91	38.44	11.30
Upper 95% CI of mean	433.5	461.4	173.5	179.2	68.09	75.68	88.70	61.56

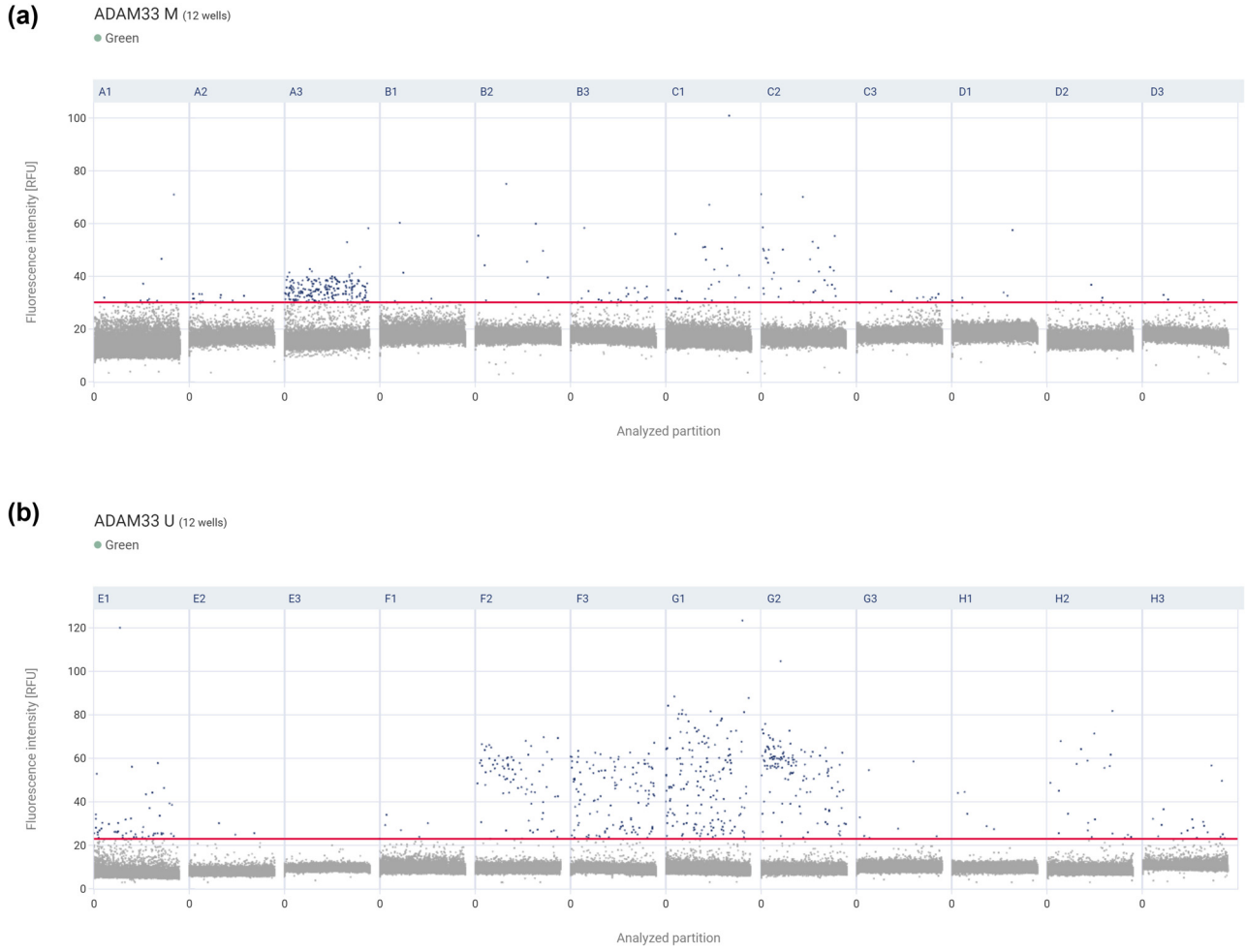

Figure 3: Fluorescence intensity of the analyzed partitions in (a) methylated and (b) unmethylated ADAM33 assays in lung samples. The red line indicates the threshold between positive and negative partitions (gray dots). *dot plot obtained from the QIAcuity 5 plex (QIAGEN) equipment showing the rainfall on the samples analyzed.

copies in both conditions, with a more pronounced increase when associated with COPD by copies/uL (p = 0.0385), but not copies/ng of DNA (Figure S2).

Analyzing the number of copies or the methylation rate in lung tissue of patients exclusively with COPD was not feasible, as it is not common to biopsy this tissue in those patients. In addition, several studies have described changes in the methylation profile preceding the appearance of many diseases, which could influence the results of our analysis since all available samples in this study were from individuals who died from COVID-19 and had no previous biopsy to compare. Graphical and statistical analyses of the copy numbers are detailed in Supplementary material 3. To compare the sample groups, the average methylation rates were calculated and plotted on bar graphs. Figure 4 shows the analysis of the methylation profile of *ADAM33* in saliva (Figure 4a) and in lung tissue (Figure 4b). It was observed that the methylation profile was very similar between the groups. In saliva, 63.9% of the *ADAM33* gene copies were methylated in the control group, 52.8% in the

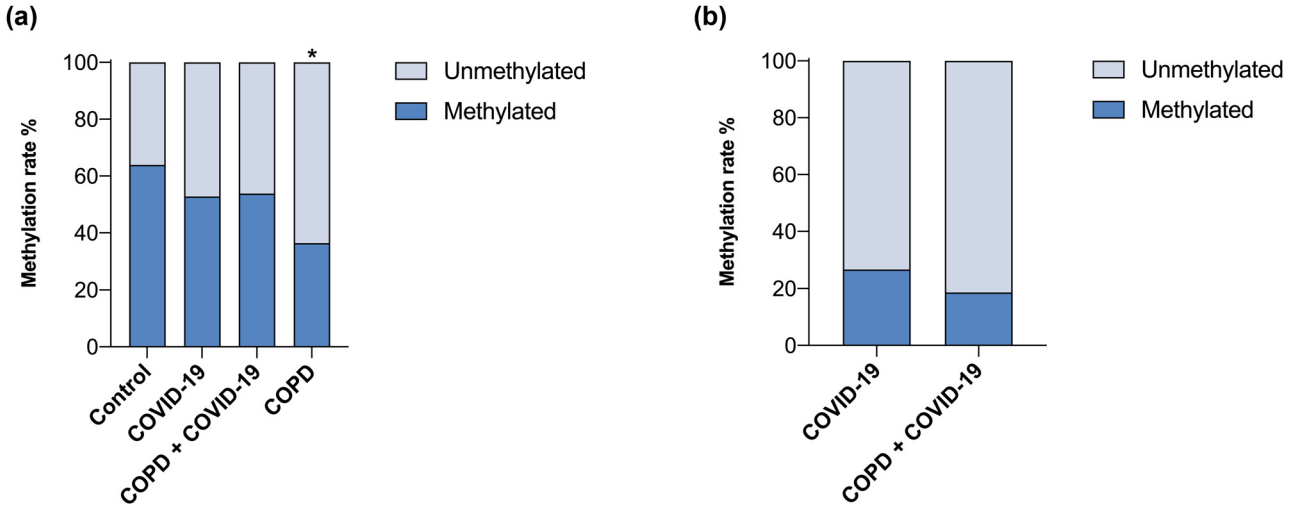

Figure 4: ADAM33 methylation rate in the control group, COVID-19 group, and/or COPD + COVID-19 group in saliva (a) and lung tissue (b). *p = 0.0001.

COVID-19 positive group, 53.8% in the COPD + COVID-19 group, and 36.4% in the COPD group. The difference between the COPD group and the control group was statistically significant (p = 0.0001). Otherwise, in the lung samples, the control group exhibited 14% of the *ADAM33* gene copies methylated, in the COVID-19 positive group, *ADAM33* displayed a 26.7% rate of methylation, while in the COPD + COVID-19 group, *ADAM33* showed 18.7% of methylation.

The confidence interval of MSP-dPCR data for ADAM33 in lung samples is described in Table 3. A negative 95% CI for some groups was observed, which may be related to the sample size, the standard deviation, and the binomial nature of the data in which high quantities of one condition result in low quantities of the other condition.

Discussion

The MSP-PCR technique is an important tool in molecular research and clinical diagnosis within the field of epigenetics. It can be used as a diagnostic tool to determine disease stage, predict treatment response, and assess the risk of recurrence [44–46]. In addition, it can be used to monitor the efficacy of epigenetic treatments, such as demethylating agent therapies, designed to reverse abnormal DNA methylation [47,48]. The identification of epigenetic biomarkers through MSP-dPCR holds potential value for disease screening, patient stratification, and personalized treatment approaches [49,50].

Asthma and COPD share similarities and significant differences. They share genetic and environmental risk factors including allergens, infections, and smoking. Notably, *ADAM33* mRNA and protein expression are restricted to mesenchymal cells, including airway fibroblasts, myofibroblasts, and smooth muscle [51,52]. In bronchial biopsies from normal and asthmatic airways, *ADAM33* colocalizes with smooth muscle and fibroblast-like cells in the submucosa, supporting its association with bronchial hyperresponsiveness and airway remodeling [53]. However, no disease-related differences in overall expression levels were identified. A study revealed that the increase of TGF-β contributed to the soluble form ADAM33 (sADAM33), leading to epithelial damage. This finding suggests a potential synergism with *ADAM33* in the pathogenesis of asthma and COPD, emphasizing the intricate interplay between genetic and environmental factors in these complex diseases [53,54].

In COPD, the *ADAM33* gene has been associated with heightened lung epithelial cell responsiveness, contributing to a poorer prognosis of the disease, this association has been linked to the methylation status of this gene [55],

Table 3: Confidence interval of MSP-dPCR data for ADAM33 in lung samples

	COVID-19 U	COVID-19 M	COPD + COVID-19 U	COPD + COVID-19 M
Lower 95% CI of mean	39.60	−6.947	24.43	−38.24
Upper 95% CI of mean	106.9	60.40	138.2	75.57

U, unmethylated; M, methylated.

leading to the hypothesis that varying methylation levels might correlate with the severity of allergic asthma, rendering it a potential target for future therapeutic and diagnostic approaches [56]. However, although *ADAM33* has been identified as a factor underlying asthma exacerbation [57], studies have shown no significant difference in CpG methylation of peripheral blood leukocytes between asthmatic adults and controls, suggesting that treatment response differs in mild and severe asthma as well as in different tissue types [58,59]. Regarding the conclusions of several studies, polymorphonuclear cells are not a well-recommended tissue type for studying the epigenetic process in asthma, unlike airway epithelial cells and nasal epithelial cells. The methylation mechanism of the *ADAM33* gene might elucidate why lung cell injury is more severe in COPD or ACO with SARS-CoV-2 infection. Our results revealed a higher percentage of methylation in saliva samples in the control group compared to other groups. When comparing individuals without respiratory disease to those in the COPD group, the ADAM33 hypermethylation rate was 63.9% compared to 36.4%, respectively. When comparing individuals without COPD who had COVID-19 or those with COPD who had COVID-19, the hypermethylation rates remained almost unchanged, at 52.8 and 53.8%, respectively. Collectively, these findings suggest that COPD individuals tend to have higher rates of unmethylated ADAM33. It is consistent with the literature that these individuals have higher expression of ADAM33, which is associated with worsened prognosis in COPD. Moreover, within the same group, we observed a decrease in the rate of unmethylation of ADAM33, from 63.6 to 46.2%, which suggests that SARS-CoV-2 infection might influence the methylation profile. However, further experiments and analyses are needed to better understand the effects of COVID-19 on DNA methylation as numerous factors could potentially alter methylation levels in these patients. These methylation rates are further supported by analyzing the number of copies between the groups, where the number of methylated copies of ADAM33 is higher in controls than in COPD patients, and conversely ($p < 0.0001$). Additional data comparing different important markers for both diseases need to be analyzed, yet our findings indicate that the percentage of the ADAM33 methylation profile in saliva could be an important diagnostic and prognostic factor for COPD severity, particularly in the context of COVID-19. Further investigation is necessary to ascertain whether this alteration might extend to other viral infections. However, this necessitates new experiments to elucidate the effects of ADAM33 methylation. It is noteworthy that despite the diversity of cell-originated DNA, methylation assessment emerges as a compelling avenue, considering the potential differentiation in methylation patterns between cells in both healthy and diseased states. However, it appears that saliva might be considered a more promising biomarker for COPD, since there were no differences between the methylation rates of ADAM33 in the lung tissue among individuals with COVID-19, regardless of COPD status. Nonetheless, additional studies are warranted to substantiate this proposition, encompassing diverse cohorts of respiratory diseases and varying biological specimens.

Considering the valuable applications of MSP-PCR in identifying diverse methylation profiles, its adaptation to the most modern available techniques is intriguing, potentially enhancing its performance. However, the MSP-dPCR technique displayed the ‘rain’ phenomenon in fluorescence intensity graphs. This occurrence might stem from the exceedingly small size of the products, approximately 50 base pairs, potentially leading to a greater influx of DNA copies within each partition compared to traditional gene expression dPCR. Another possible situation is that bisulfite conversion causes fragmentation of DNA due to harsh conditions [60]. It was described that bisulfite-converted DNA may produce a “rain” profile in dPCR, resulting in poor resolution [61]. Then, this is another possible reason why there are some poor resolutions in our results. Consequently, this could elevate the probability of equipment misinterpretation between negative and positive partitions, owing to the heightened likelihood of both methylated and non-methylated copies cohabiting within the same partition. Nonetheless, dPCR remains an excellent application option for MSP-PCR due to its advanced features that offer several advantages compared to previous generations of PCR. Apart from offering high sensitivity and precision, dPCR is less sensitive to inhibitors that can affect the results, as observed in qPCR, making it suitable for complex or poor-quality samples [50].

In summary, the MSP-dPCR technique offers specificity and precision in analyzing DNA methylation profiles. To achieve this, it is crucial to emphasize that the dPCR and the primers for the unmethylated and methylated conditions must be carefully standardized and validated, following the guidelines for dPCR [2,51,52]. Moreover, the utilization of normalizing genes might not be necessary in the specific case of analyzing percentages of unmethylated and methylated conditions of the same sample by MSP-dPCR, which facilitates data analysis. Using the MSP-dPCR technique, this study evaluated for the first time the methylation profile of *ADAM33* from saliva and lung tissue samples, establishing connections to COPD and COVID-19 diseases. Therefore, the combination of MSP-PCR and dPCR presents an opportunity for even more robust and accurate results in DNA methylation studies in across several pathologies.

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Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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