Combined use of rheology and portable low-field NMR in cystic fibrosis patients

Michela Abrami a, Massimo Maschio b, Massimo Conese c, Marco Confalonieri d, Fabio Gerin a, Barbara Dapas e, Rossella Farra f, Alessandra Adrover g, Lucio Torelli f, Barbara Ruaro d, Gabriele Grassi e,*, Mario Grassi a

a Department of Engineering and Architecture, University of Trieste, Via Valerio 6/A, I-34127, Trieste, Italy
b Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Via Dell’Istria, 65, I-34137, Trieste, Italy
c Department of Medical and Surgical Sciences, Foggia University, Ospedali Riuniti, Via L. Pinto, 1, I-71122, Foggia, Italy
d Cattinara University Hospital, Pulmonology Department, Strada di Fiume 447, I-34149, Trieste, Italy
e Department of Life Sciences, Cattinara University Hospital, Trieste University, Strada di Fiume 447, I-34149, Trieste, Italy
f Clinical Department of Medical, Surgical and Health Sciences, Cattinara University Hospital, Trieste University, Strada di Fiume 447, I-34149, Trieste, Italy
g Department of Chemical Engineering, Materials and Environment, Sapienza University of Roma, Via Eudossiana 18, I-00184, Rome, Italy

ARTICLE INFO

Keywords:
Cystic fibrosis
Rheology
Low field NMR
Sputum

ABSTRACT

Background: As most cystic fibrosis (CF) patients progress to respiratory failure, lung functionality assessment is pivotal. We previously developed a test that indirectly monitors airways (inflammation-functional test) by measuring the spin-spin relaxation time ($T_{2m}$) of the water hydrogens present in CF sputum. Here the $T_{2m}$ significance in the monitoring of CF lung disease was further investigated by studying the correlation of $T_{2m}$ with: 1) sputum viscoelasticity, 2) mucociliary clearability index (MCI)/cough clearability index (CCI) and 3) sputum average mesh-size.

Methods: Sputum samples from 25 consenting CF subjects were analyzed by rheology tests (elastic modulus $G$ and zero shear viscosity $\eta_0$) and Low Field Nuclear Magnetic (LF-NMR) resonance ($T_{2m}$). MCI/CCI were calculated from the rheological parameters. The average mesh-size ($\xi$) of the sputum structure was then evaluated by rheology/LF-NMR, together with FEV$_1$ for each patient.

Results: There was an inverse correlation between $G$ and $\eta_0$ versus $T_{2m}$, indicating that a worsening of the lung condition ($T_{2m}$-FEV$_1$ drop) is paralleled by an increase in sputum viscoelasticity ($G$ and $\eta_0$) favoring mucus stasis/inflammation. A direct correlation was also observed between $T_{2m}$ and MCI/CCI, showing that $T_{2m}$ provides information as to airway mucus clearing. Moreover, there was a direct correlation between $T_{2m}$ and the average sputum mesh size ($\xi$).

Conclusions: We demonstrated a correlation between $T_{2m}$ (measured in CF patient’s sputum) and the sputum viscoelasticity/average mesh-size and with MCI/CCI, parameters related to airway mucus clearing. Thus, the present data strengthen the potential of our test to provide indirect monitoring of airway disease course in CF patients as $T_{2m}$ depends on mucus solid concentration and nanostructure.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding of the cystic fibrosis transmembrane conductance regulator (CFTR) [1,2] responsible for chloride and sodium ion exchange across epithelial membranes. Dysfunctional CFTR induces the production of thick/viscous mucoid secretions in multiple organs, in particular the airways, where an augmented mucus viscosity [3,4] is determined by the pathological increase in proteins, mucin and biological polymers [5]. This process impairs mucociliary clearance, promoting inflammation and bacterial infection [6]. Chronic inflammation can lead to airway remodeling, which, in turn, may progress to respiratory failure, the most common cause of death for CF patients [7]. Therefore, as sputum is a rich and noninvasive source of biomarkers of inflammation/infection, the determination of sputum properties is a relevant parameter able to indirectly monitor lung disease.

* Corresponding author.
E-mail address: ggrassi@units.it (G. Grassi).
Recently [8,9], we studied the characteristics of CF sputum using low field nuclear magnetic resonance (LF-NMR). Expected sputum is easily obtained from patients and its properties mirror those of mucus [5,10]. The LF-NMR technique we used allows for the measurement of the spin-spin relaxation time ($T_{2m}$) of the water hydrogens present in the CF sputum. Our previous data showed [8,9] that the $T_{2m}$ measured in CF sputum has an indirect correlation with circulating/local inflammation markers and a direct correlation with FEV1 (forced expiratory volume in the first second, i.e. the amount that is exhaled in the first second purposefully trying to breath out as much air as possible), the most commonly used test in clinical practice for the evaluation of lung function [11]. Together, our previous findings, suggest that the assessment of physical properties of sputum by $T_{2m}$ may well provide a useful tool for the indirect monitoring of lung disease in CF patients. Indeed, as it will be discussed in this paper, $T_{2m}$ can provide information about sputum nano-structure that the determination of solid concentration cannot.

The main objective of this study was to further investigate the role $T_{2m}$ plays in the monitoring of lung disease in CF patients. To this aim investigations were carried out to determine as to any possible correlation between $T_{2m}$ and sputum viscoelasticity as several authors have reported a relationship between sputum viscoelasticity and the severity of lung pathology [12–17]. Moreover, Odeblad et al. used LF-NMR and rheology to study the properties of lung mucus [18,19].

A secondary objective was that of investigating whether there was any correlation between $T_{2m}$ and both the mucociliary clearability index (MCI) and the cough clearability index (CCI). MCI and CCI, which can be calculated from the rheological properties in sputum, as reported by King et al. [20,21], represent the two main mucus clearance mechanisms. In healthy subjects, proper MCI/CCI allows for mucus clearance from the airways, preventing mucus stasis and, consequently, bacterial infection/inflammation. In CF subjects, the reduction in MCI/CCI is a relevant finding as it reflects the severity of lung pathology. In this regard, Ma et al. [13], studied the rheology of CF sputum and measured mucociliary clearability and cough clearability showing that sputum viscoelastic properties are tightly associated with lung function and disease status. The study of Ma et al. was lead on a per patient basis and a trend in a cross-population analysis was not found.

As last objective of this work, relying on the Flory theory [22] and on the rheological data, we estimate the sputum average mesh size $\xi$, a parameter useful to predict drug diffusion (and thus effectiveness) through mucus whose permeability to drugs is known to decrease with its viscoelastic properties [23].

Therefore, in this work, the possible correlation between $T_{2m}$ and sputum viscoelasticity/MCI/CCI/sputum-average mesh size was assessed, for the first time, by a combined analysis based on rheology and LF-NMR. This novel approach provided information as to the macro- (viscosity, elasticity, average magnetic relaxation time) and micro-scale (mesh size distribution) characteristics of CF sputum.

2. Materials and methods

2.1. Sample collection

Sputum samples from 25 CF patients (a mix of clinical status) were provided by the Burlo Garofolo Hospital, following a procedure approved by the Ethics Committee (prot n. 496/2916, CI M –11, 22-3-2016). Written informed consent was obtained from each patient. One sputum specimen was collected before a chest physiotherapy session in randomly selected adult and young patients (see Table 1) able to expectorate. Non-expectorant patients were excluded, as were subjects <7 years of age. The only exception was patient n.10 (Table 1) where three samples were taken into consideration so as to study the time evolution over a 3 months period. However, only the first sample was considered in the statistical analyses. The samples of healthy subjects reported in Fig. 1 were obtained from published papers [24].

So as to evaluate any correlation among the sputum shear modulus $G$, zero-shear viscosity $\eta_0$, average magnetic relaxation time $T_{2m}$ and FEV1 in a broad range of FEV1 values, patients with a large uniform FEV1 distribution (from $\approx$31% to 85%, see also Table 1) were selected. The aforementioned criteria allowed for the recruitment of most patients who attended Burlo Garofolo Hospital for a clinical visit during our study period (from May 2019 to June 2020). Spontaneously expectorated (1–2 ml) sputum was collected from CF patients in sterile cups and immediately used for $T_{2m}$ determination. The samples were then transferred from the LF-NMR glass tube to a rheometer device for analysis.

<table>
<thead>
<tr>
<th>n°</th>
<th>I mut</th>
<th>II mut</th>
<th>sex</th>
<th>age</th>
<th>FEV$_1$</th>
<th>Therapies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F508del</td>
<td>N1303K</td>
<td>M</td>
<td>42</td>
<td>80</td>
<td>minocycline + levofloxacin</td>
</tr>
<tr>
<td>2</td>
<td>F508del</td>
<td>IS507del</td>
<td>M</td>
<td>36</td>
<td>64</td>
<td>colistimethate</td>
</tr>
<tr>
<td>3</td>
<td>F508del</td>
<td>711-5G-&gt;A</td>
<td>M</td>
<td>44</td>
<td>61</td>
<td>ciproxin + prednisone</td>
</tr>
<tr>
<td>4</td>
<td>F508del</td>
<td>F508del</td>
<td>F</td>
<td>15</td>
<td>82</td>
<td>azithromycin + prednisone + minocycline</td>
</tr>
<tr>
<td>5</td>
<td>G542X</td>
<td>delta507</td>
<td>F</td>
<td>18</td>
<td>85</td>
<td>colistin + azithromycin</td>
</tr>
<tr>
<td>6</td>
<td>F508del</td>
<td>2,3del(21 kb)</td>
<td>M</td>
<td>37</td>
<td>79</td>
<td>prednisone</td>
</tr>
<tr>
<td>7</td>
<td>F508del</td>
<td>1677delTA</td>
<td>M</td>
<td>34</td>
<td>62</td>
<td>Minocin + ciprofloxacin</td>
</tr>
<tr>
<td>8</td>
<td>G542X</td>
<td>2347delG</td>
<td>M</td>
<td>36</td>
<td>79</td>
<td>levofloxacin</td>
</tr>
<tr>
<td>9</td>
<td>F508del</td>
<td>3659delC</td>
<td>F</td>
<td>49</td>
<td>47</td>
<td>No antibiotic therapy</td>
</tr>
<tr>
<td>10</td>
<td>F508del</td>
<td>F508del</td>
<td>F</td>
<td>23</td>
<td>34</td>
<td>levofloxacin</td>
</tr>
<tr>
<td>11</td>
<td>1717-1G-&gt;A</td>
<td>1717-1G-&gt;A</td>
<td>M</td>
<td>27</td>
<td>71</td>
<td>prednisone + azithromycin + ceftazidime</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>M</td>
<td>67</td>
<td></td>
<td>amikacin + imipenem + cefoxitin</td>
</tr>
<tr>
<td>13</td>
<td>F508del</td>
<td>3120+1kbdel8,6k</td>
<td>M</td>
<td>35</td>
<td>59</td>
<td>levofloxacin</td>
</tr>
<tr>
<td>14</td>
<td>F508del</td>
<td>F508del</td>
<td>F</td>
<td>33</td>
<td>31</td>
<td>prednisone + ciprofloxacin + minocycline + itraconazole + aerosol amikacin</td>
</tr>
<tr>
<td>15</td>
<td>G542X</td>
<td>G542X</td>
<td>F</td>
<td>31</td>
<td>65</td>
<td>prednisone + levofloxacin</td>
</tr>
<tr>
<td>16</td>
<td>F508del</td>
<td>F508del</td>
<td>M</td>
<td>21</td>
<td>38</td>
<td>prednisone + ciprofloxacin + minocycline + itraconazole + aerosol amikacin</td>
</tr>
<tr>
<td>17</td>
<td>F508del</td>
<td>F508del</td>
<td>F</td>
<td>37</td>
<td>67</td>
<td>levofloxacin</td>
</tr>
<tr>
<td>18</td>
<td>N1303K</td>
<td>G542X</td>
<td>M</td>
<td>27</td>
<td>37</td>
<td>levofloxacin + prednisone + colistimethate</td>
</tr>
<tr>
<td>19</td>
<td>F508del</td>
<td>N1303K</td>
<td>M</td>
<td>34</td>
<td>43</td>
<td>prednisone + ciprofloxacin + minocycline + itraconazole + tobramycin</td>
</tr>
<tr>
<td>20</td>
<td>F508del</td>
<td>F508del</td>
<td>F</td>
<td>25</td>
<td>46</td>
<td>temocillin + amikacin + prednisone + sodium colistimethate</td>
</tr>
<tr>
<td>21</td>
<td>F508del</td>
<td>4382delA</td>
<td>F</td>
<td>56</td>
<td>57</td>
<td>Bactrim (sulfamethoxazole + trimethoprim ratio 5:1)</td>
</tr>
<tr>
<td>22</td>
<td>F508del</td>
<td>F508del</td>
<td>F</td>
<td>26</td>
<td>66</td>
<td>levofloxacin</td>
</tr>
<tr>
<td>23</td>
<td>R1162X</td>
<td>1717-1G-&gt;A</td>
<td>M</td>
<td>43</td>
<td>40</td>
<td>prednisone + Bactrim + levofloxacin</td>
</tr>
<tr>
<td>24</td>
<td>F508del</td>
<td>F508del</td>
<td>M</td>
<td>43</td>
<td>41</td>
<td>prednisone + ciprofloxacin + sodium colistimethate</td>
</tr>
<tr>
<td>25</td>
<td>F508del</td>
<td>2368-69del11</td>
<td>F</td>
<td>20</td>
<td>49</td>
<td>ciprofloxacin + prednisone</td>
</tr>
</tbody>
</table>
Rheology

Rheology is used to describe and assess the flow behavior of materials. Fluids flow at different speeds and solids can be deformed to a certain extent. Depending on their physical behavior, materials can be put into liquids on one side and solids on the other. Rheological measurements, including viscosity (resistance to flow) and elasticity (stiffness), are often used together to describe the consistency of mucus. The rheological properties of mucus vary as a function of shear stress, time scale (rate) of shearing, and length scale. Changes in the rheological properties of mucus may greatly affect its ability to function as a lubricant, selective barrier, and the body’s first line of defense against infection. In addition, the rheological characterization can provide insight into the nanostructure of a gel-like material such as mucus/sputum.

The viscoelastic properties of the analyzed samples were determined by three typical rheological tests, i.e. the stress sweep, frequency sweep and steady value test. While stress sweep tests serve to detect the maximum deformation the sample can undergo without altering its internal micro-nano-structure, frequency sweep tests determine how much the elastic ($G'$) and viscous ($G''$) properties (moduli) of the sample depend on solicitation pulsation $\omega$. Relying on $G'$ and $G''$ knowledge, the shear modulus $G$ of the sample was determined as previously described [25,26] and as detailed in supporting information S1. Then, knowing $G$, Flory theory [22] and the equivalent network theory [27] allow to evaluate the average mesh size $\xi$ of the polymeric network pervading CF sputum as explained in supporting information S1.

Interestingly, some insights as to lung functionality/inflammation can be deduced by the evaluation of the mucociliary clearability index (MCI) and the cough clearability index (CCI) [28–30] (see also supporting information S1 for further details on the determination of MCI and CCI by rheological characterization). Indeed, as many of the particles that settle on airway surfaces are infectious, airways have evolved innate defense mechanisms that constantly protect them against bacterial and other types of infection. The two major mucus clearance mechanisms from the airways are mucociliary clearance and, when it fails or is overloaded, coughing [31]. As the cilia beat at about 10–20 Hz, with an amplitude of 5 $\mu$m, mucus transport velocity (frequency $\times$ amplitude) is in the order of 1 cm/min, whilst the velocity during a cough maneuver is about 100 times greater. Therefore, low-frequency (1 rad/s) viscoelastic measurements are appropriate for observations of mucociliary clearance, whereas high-frequency measurements (100 rad/s) are more predictive for cough clearance [32–34].

Other important information can be obtained from the steady value test (SV) that assesses the dependence of the sample shear viscosity ($\eta$) on the shear rate ($\dot{\gamma}$). The sample viscosity, evaluated in correspondence to a vanishing shear rate (zero shear viscosity $\eta_0$), is a particularly important parameter. $\eta_0$ was determined by fitting the Cross model [35] onto the SV data (see also supporting information S1). All rheological measurements were performed in triplicate by a stress controlled rotational rheometer (Haake Mars Rheometer, 379-0200 Thermo Electron GmbH, Karlsruhe, Germany) equipped with cone-plate geometry (C35, diameter $= 35$ mm). So as to avoid problems of evaporation, sputum characterization was done at $+20$ $^\circ$C, using a glass solvent trap.

2.3. Portable low field NMR

NMR relies on the ability of hydrogen atoms dipole to react to the perturbation of an external constant magnetic field $B_0$ where they are embedded. Basically, after a temporary $B_0$ perturbation obtained by the application of a radio frequency pulse $B_1$, perpendicular to $B_0$, the dipoles tend to return to their initial alignment with $B_0$ (relaxation). Relaxation rate is expressed by the inverse of a characteristic time constant $T_2$ called spin-spin or transverse relaxation time [36]. The relaxation process (signal amplitude decay $I(t)$, $t = \text{time}$) can be described by the sum of $m$ exponential terms, each one characterized by a different time decay constant $(T_{2i})$ and weight $(A_{2i} = A_{1i} n_{i} + A_{2i} n_{j} + \ldots + A_{m_{i}} = 100{\%})$ [37]. This allows for the evaluation of the average relaxation time $(T_{2m})$ and the inverse relaxation time ($(1/T_{2m})$, as detailed in supporting information S2. Interestingly, at each relaxation time $T_{2i}$ corresponds a mesh of size $\xi_{i}$, as reported in supporting information S2 and in Refs. [36–38].

LF-NMR measurements were taken at $+37$ $^\circ$C, by a Bruker Minispec mq20 (0.47 T, Germany, Figure S1). The $T_{2m}$ determination was performed according to the CPMG sequence (Carr–Purcell–Meiboom–Gill) [39] ($90^\circ - \tau - 180^\circ - \tau - \text{echo})n-T_{2}$) with a 8.36 $\mu$s wide 90$^\circ$ pulse, $\tau = 250$ $\mu$s and a $T_{E}$ (sequence repetition rate) equal to 5 s. Each spin-echo decay had $n$ points and was repeated 36 times (number of scans).

Fig. 1. Viscosity ($\eta$) dependence on shear rate ($\dot{\gamma}$) referring to our CF sputum samples (black lines) and literature data referring to healthy human mucus (grey circles) [40] and CF sputum (white diamonds) [24] evaluated at physiological shear rates (healthy lung [24] and the maximum shear rate reached during coughing [40]).
2.4. Statistical analysis

The nature of the experimental data distribution (normal or not) was assessed by the Kolmogorov-Smirnov test (KS-test). Based on the KS-test results, the Spearman’s correlation coefficient ($r_{sp}$) verified direct or inverse correlations amongst $G'$, $\eta_0$, $T_{2m}$, $FEV_1$, $MCI$ and $CCI$. Any p-values below 0.05 were considered statistically significant.

Principal component analysis (PCA) on the variance/covariance matrix as well as on the correlation matrix was performed to assess the strength of the correlation among the different variables. 95% confidence ellipse from the covariance matrix of bivariate data was evaluated to better visualize direct or inverse correlations and to identify bivariate outliers. The axes of the ellipse are oriented along the eigenvectors of the covariance matrix and their lengths are proportional to the square root of the corresponding eigenvalues times a factor 1.96, corresponding to a 95% confidence interval for a normal distribution.

3. Results

3.1. Rheological and LF-NMR characterization of sputum

The flow properties of the CF sputum collected in this study were compared to those of healthy subjects’ sputum and patients obtained from published papers. Fig. 1 shows the comparison among the viscosity (flow curves, black lines) of our CF sputum samples with the viscosity of healthy mucus (grey circles) [40] and CF sputa (white diamonds) obtained from previously published data [24] and evaluated in correspondence to typical shear rates ($\dot{\gamma}$) in healthy lungs ($\dot{\gamma} = 10^{-10^0} 1/s$) [24] and during coughing ($\dot{\gamma}_{max} = 10^3 - 10^4 1/s$) [40].

The viscosity of the CF sputa was considerably higher than that of healthy mucus across all the shear rate range, in agreement with literature data (white diamonds) [24].

The frequency sweep tests, shown in Fig. 2, relate to three representative samples and show the gel-like nature (or incipient gel nature) of our samples. Indeed, the data show that the elastic component ($G'$) prevailed over the viscous one ($G''$) and both $G' - G''$ were almost independent of pulsation $\omega$ [25,26]. Healthy subjects’ sputa essentially coincides with saliva, characterized by $G' \approx 0$. Sputum characterization was completed by LF-NMR analysis (Fig. 3). The data showed a signal amplitude decay ($I(t)$) for five representative samples and one healthy subject. There are evident differences in the decay curves, which depend on the increased concentration and spatial organization of the solids within the sputum. This, in turn, determines a higher sputum dehydration [8,9] and viscosity.

3.2. The relationship of the sputum rheological features with $T_{2m}$ and $FEV_1$

Fig. 4A shows the inverse correlation between both the shear modulus $G'$ (elastic properties) and the zero shear viscosity $\eta_0$ (viscous properties) compared to $T_{2m}$. There is an evident link between disease worsening (a drop in $T_{2m}$) and the increased elastic and viscous properties of the sputum. Also $FEV_1$ had an inverse correlation with $\eta_0$ and $G'$ (Fig. 4B). Notably, the $\eta_0$ correlation with $T_{2m}$ and $FEV_1$ (Fig. 4A and B) is less pronounced than that observed with $G'$, this can be appreciated by the direct comparison between the 95% confidence ellipses. Moreover, the data extrapolated from Fig. 4A and B confirm our previous findings [8,9], i.e. there is a significant correlation ($r_{sp} = 0.55$, $p = 4*10^{-3}$) between $T_{2m}$ and $FEV_1$ (correlation line: $FEV_1 = (1.7 \pm 0.5)*10^{-2}*T_{2m} + (40 \pm 6)$).

3.3. The relationship between $MCI$ and $CCI$ with $T_{2m}$ and $FEV_1$

$MCI$ and $CCI$, which can be calculated from the rheological properties of the sputum [28-30], reasum the sputum viscoelastic features. Fig. 5A shows a direct correlation between $T_{2m}$ and $CCI/MCI$. Our data also showed a direct correlation between $CCI/MCI$ and $FEV_1$ (Fig. 5B), suggesting that the progressive deterioration of lung function (decline in $FEV_1$) occurred in parallel with a reduced mucus clearance ($CCI/MCI$) ability.

3.4. The determination of the average mesh size of sputum

The synergic combination of rheology and LF-NMR allows for an estimation of a very important parameter, i.e., the average mesh size ($\xi$) of the polymeric structure pervading the sputum of CF patients. It is well known that drug diffusivity inside a gel-like system depends on the ratio between the diffusing drug molecule radius and $\xi$ [41]. The smaller this ratio, the higher the drug accessibility into mucus, enhancing its efficacy. Relying on what is presented in sections 2.2-2.3 and in supporting information S2, Fig. 6 shows the direct correlation between $\xi$ and $T_{2m}$.

![Fig. 2. Frequency sweep test. Elastic ($G'$ - full symbols) and viscous ($G''$ - empty symbols) modulus dependence on pulsation ($\omega = 2\pi f$) at $\tau = 0.05$ Pa ($T = 20$ °C) referring to three representative samples. Braces help in enucleating each mechanical spectrum, i.e, the $G'$ and $G''$ trends corresponding to a single patient. All the other samples fall within the values shown in the picture and are not reported to keep a good image clarity.](image)

![Fig. 3. Transverse magnetization reduction ($I(t)$) referring to five representative sputum samples from five CF patients (dashed/dotted lines), plus one referring to a healthy subject (solid line). All the other samples fall within the values shown in the picture and are not reported to keep a good image clarity.](image)
Interestingly, Fig. 6 indicates that, in most cases (92.5%), ξ spans from about 50 nm to 150 nm, a range in line with literature data on CF sputum (∼60–300 nm with average size (140 ± 50) nm) [42,43]. Notably, the combination of rheology and LF-NMR is not limited to ξ evaluation as it also allows for the determination of the mesh size distribution (AΨ, ξ) as depicted in Fig. 7. Indeed, assuming that the three-dimensional network pervading the whole sputum is composed by meshes of different size ξ, it can be demonstrated [36–38] that at each mesh size ξi corresponds the relaxation time \( T_{2i} \) (see also supporting information S2):

\[
ξ_i = ξ \left( \frac{1}{T_{2i}} - \frac{1}{T_{2m}} \right)
\]

where \( T_{2m} \) is the relaxation time of bulk water hydrogen (i.e. water far from polymer chains; \( T_{2m} \approx 3700 \text{ ms at } 37\,\text{°C, } 20\,\text{ MHz} \) [44]) and ξ indicates the average mesh size determined by means of rheology. As detailed in the Supporting Information S2, \( T_{2i} \) and the relative % abundance AΨ, are determined by fitting the experimental relaxation data (see Fig. 3) by means of a sum of exponential terms (AΨ*exp(-ξi/T_{2m})/t) is time).

Fig. 7 refers to a single patient (n. 10, Table 1) for which we have collected three sputum samples over a period time of three months at hospitalization and discharge. At first hospitalization (day 0), 17% of the meshes were 211 nm, 55% 61 nm and 28% 24 nm (see horizontal black bars in Fig. 7). Thirty days later (discharge), after antibiotic therapy, the percentage of day 0 meshes was 39%, with a parallel reduction in \( T_{2i} \) from 300 nm with average size (140 ± 50) nm) [42,43]. Altogether, these modifications led to an overall increase in sputum mesh size, which can justify the \( T_{2m} \) increase as expected from the data reported in Fig. 6. Notably, also FEVI improved, as expected judging from the \( T_{2m} \) increase.

Fig. 4. A) There is a significant inverse correlation between the shear modulus \( G \) (white circles; left vertical axis, see the left oriented arrow) and the average relaxation time \( T_{2m} \) (\( r_p = -0.57, p = 3 \times 10^{-5} \)). The dashed line indicates the linear interpolant \( \eta \) (black circles; right vertical axis, see the right oriented arrow) and the average relaxation time \( T_{2m} \) (\( r_p = -0.48, p = 1.7 \times 10^{-5} \)). The dashed line indicates the linear interpolant \( \eta_0 \) (black circles; left vertical axis, see the left oriented arrow) and the average relaxation time \( T_{2m} \) (\( r_p = 0.36, p = 4.6 \times 10^{-5} \)). B) There is a significant inverse correlation between the shear modulus \( G \) (white circles; left vertical axis, see the left oriented arrow) and the average mesh size \( ξ \) (black circles; right vertical axis, see the right oriented arrow) and \( T_{2m} \) (\( r_p = -0.63, p = 0.8 \times 10^{-5} \)). The dashed line indicates the linear interpolant \( \eta \) (black circles; right vertical axis, see the right oriented arrow) and \( T_{2m} \) (\( r_p = -0.41, p = 4.6 \times 10^{-5} \)).

Fig. 5. A) There is a significant direct correlation between \( CCI \) (black circles; left vertical axis, see the left oriented arrow) and the average relaxation time \( T_{2m} \) (\( r_p = 0.56, p = 2.3 \times 10^{-4} \)). linear interpolant (dashed line) \( CCI = (6.7 \pm 3.0) \times 10^{-3}T_{2m} + (21 \pm 50) \) nm) [42,43]. B) There is a significant direct correlation between \( CCI \) (black circles; left vertical axis, see the left oriented arrow) and \( FEV_1 \) (\( r_p = 0.68, p = 2.0 \times 10^{-4} \)). linear interpolant (dotted line) \( CCI = (3.5 \pm 0.9) \times 10^{-3}FEV_1 + (0.7 \pm 0.3) \). B) There is a significant direct correlation between \( MCI \) (white circles; right vertical axis, see the right oriented arrow) and \( T_{2m} \) (\( r_p = 0.5, p = 1.2 \times 10^{-2} \)). linear interpolant (dashed line) \( MCI = (4.5 \pm 1.7) \times 10^{-3}FEV_1 + (0.73 \pm 0.1) \). The solid lines indicate the 95% confidence ellipses.
The time evolution of the mesh size distribution \( (\xi, \text{black circles}) \) of the sputum polymeric network and the average relaxation dience ellipses. In order to deeply understand the potentiality of patients, as respiratory failure is the leading cause of death in these subjects [7]. In order to investigate which of the measured parameters among \( G \) and \( T_{2m} \) possesses the strongest linear correlation with \( FEV_1 \) (Figs. 4 and 5) a bivariate Principal Component Analysis (PCA) was performed as detailed in Supporting Information S3. The results of PCA can be summed up by the correlation coefficient \( p_{X,Y} \). This analysis reveals that \( G (p_{PREV1,G} = -0.576) \) and \( T_{2m} (p_{PREV1,T2m} = 0.57) \) exhibit a strong similar correlation with \( FEV_1 \) while a weaker correlation between \( \eta_0 \) (\( p_{PREV1,\eta_0} = -0.162 \)) and \( FEV_1 \) is observed. The same analysis, performed on \( MCI \) and \( CCI \) data, indicates a strong direct correlation between \( MCI - FEV_1 \) (\( p_{PREV1, MCI} = 0.494 \)) and \( CCI - FEV_1 \) (\( p_{PREV1,CCI} = 0.632 \)). Thus, among all the considered parameters, \( CCI \) exhibits the best correlation with \( FEV_1 \). These findings are also confirmed by ranking the correlation between
The third important finding in the present investigation is the determination of the correlation between T_{2m} and the average mesh size \( \xi \). Indeed, this parameter has long attracted the attention of researchers due to its relevance on the lung function of a patient. Thus, the determination of a direct correlation between T_{2m} and the average mesh size (\( \xi \)) of the polymeric network pervading the sputum is also relevant from a clinical point of view (Fig. 6). This correlation is in line with the concept that T_{2m} is not only dependent on sputum polymer concentration [9, 8] but also on the three-dimensional organization of the sputum polymeric network (typically represented by mucins) that determines \( \xi \). This observation may influence therapy as the concentration/spatial organization of pathological substances in the sputum may significantly impair drug efficacy by creating a sort of “shield”. Therefore, patients with very low T_{2m} value may well benefit from the administration of muco-active agents. It is important to remind that, theoretically, the determination of \( \xi \) could be also performed according to the approximated Scherer theory [45] developed by Abrami [38]:

\[
\xi = R_0 \sqrt{\frac{C_1}{C_0} 1 - 0.58\phi \phi}
\]  

(4)

while \( R_0 \) is the radius of the polymeric chains constituting the sputum nanostructure, imagined as an ensemble of interconnected long cylindrical fibers. However, the complex conformation of the different polymeric chains makes very difficult the correct estimation of \( R_0 \). Thus, in the case of sputum, we believe that \( \xi \) determination is more safely performed by means of rheology (see Supporting Information S1, eqs. S1.3 and S1.4).

Although we only reported one patient with a 3 month follow-up, our data suggest that the different component (A_1 - A_2 and A_3) of the mesh size (\( \xi \)) can vary over time (Fig. 7). However, further studies are necessary before solid conclusions can be drawn from this preliminary observation, even if our data suggest that the increase in the A_1 component along with the decrease in A_2/A_3 is responsible for T_{2m}/FEV_1 improvement.

As only a small volume of sputum, on average 1–2 ml was tested, it may be that the samples do not accurately reflect the entire lung status. However, some considerations argue against this possibility. Firstly, in line with our previous studies [9] where more than 100 samples were analyzed, a direct correlation between T_{2m}/FEV_1 and T_{2m}/rheological properties of the sputum (Fig. 4A) was observed. This correlation would not be evident if the sample were non-representative. Secondly, the quantity of sputum sampled is in line with that analyzed in recent studies comparing the rheological properties of CF sputum with FEV_1 [13,34]. Lastly, it must be remembered that a larger sputum volume cannot be realistically obtained from a patient.

5. Conclusions

T_{2m} measurement is more practical than the widely used FEV_1 to monitor CF patients as FEV_1 is highly technique- and effort-dependent. Moreover, compared to FEV_1, T_{2m}: 1) reflects systemic and local inflammation as we have recently reported [8,9]; 2) correlates with sputum viscoelasticity and MCI/CCI/sputum average mesh-size (this paper), 3) is a quick procedure, i.e., it requires less than 1 min per sample, 4) no special operator training is required, 5) it is very inexpensive (a very good cost/benefit ratio). Therefore, T_{2m} measurement can be performed during routine visits, enabling the clinician to take real time decisions. In addition, T_{2m} shows an important advantage over the determination of the sputum solid concentration. T_{2m} is not only affected by solid concentration (as experimentally demonstrated in our previous paper [8]) but it also depends on the three-dimensional organization of the sputum solid components as theoretically explained at the beginning of the Discussion section. Thus, T_{2m} determination allows to get a picture of the sputum nanostructure that cannot be obtained by the determination of solids concentration.

In conclusion, to the best of our knowledge, this is the first study to use both macro-rheological and LF-NMR data in the characterization of CF sputum micro- and macro properties. The results herein reported, together with our previous findings, strengthen the significance and the potential utility of T_{2m} for an easy and fast indirect monitoring of lung disease in CF patients.

CRediT authorship contribution statement

Michela Abrami: Execution of experimental tests, data fitting and analysis. Massimo Maschio: selected the patients, performed FEV1 measurement, provided the clinical data of the patients selected. Massimo Conese: originally proposed the research subject, critically discussed the overall data obtained. Marco Confalonieri: made available his experience in the pneumology field to discuss the data obtained particularly in relation to the practical use/utility of the method. Fabio Gerin: sample data, transferring the samples to the research lab, Formal analysis. Barbara Dapas: Formal analysis. Rossella Farra: Formal analysis. Alessandra Adrover: Formal analysis. Lucio Torelli: general planning of the work, coordinated all the activities, Writing – original draft. Barbara Ruaro: made available her experience in the pneumology field to discuss the data obtained particularly in relation to the practical use/utility of the method. Gabriele Grassi: general planning of the work, coordinated all the activities, Writing – original draft. Mario Grassi: general planning of the work, coordinated all the activities and, Writing – original draft.

Declaration of competing interest

All the authors declare that no conflicts of interest exist.

Acknowledgments

This work was supported by the Società Italiana di Reologia – SIR – Italy, by Fondazione Cassa di Risparmio di Trieste, Italy (# 10.3069), and by the so-called “Programma di valorizzazione dei brevetti del sistema universitario del Friuli Venezia Giulia - FVG PoC, 2020, Italy.

References
