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Research article

# Comparative analysis of genetic variants in pleural fluids and solid tissue biopsies of pleural mesothelioma patients: Implications for molecular heterogeneity assessment☆

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ABSTRACT

*Objectives:* This study aims to determine whether the sequencing of DNA extracted from pleural fluids (PFs) of Pleural Mesothelioma (PM) patients accurately represents the genetic information obtained from the solid tissue counterpart biopsies with particular attention to the identification of single nucleotide variants (SNVs).
*Materials and methods:* Single pleural biopsy, PFs, and blood were collected from PM patients. DNA was extracted from these samples and then subjected to Whole-Exome Sequencing.
*Results:* A higher number of SNVs was identified in PFs than in solid tissue biopsies (STBs). Most SNVs were detected in PFs samples but not in STBs samples, while only a few SNVs were detected in STBs samples but not in PFs samples.
*Conclusion:* The current findings support the notion that PFs might offer a more robust depiction of cancer's molecular diversity. Nonetheless, the current outcomes challenge the assertion that liquid biopsies can encompass the entirety of intra-patient variations. Indeed, a subset of potential cancer-driver SNVs was exclusively identified in STBs. However, relying solely on STBs would have precluded the detection of significant SNVs that were exclusively present in PFs. This implies that while PFs serve as a valuable complement to STBs, they do not supplant them.

## 1. Introduction

Pleural mesothelioma (PM) is a rare and aggressive cancer arising from the pleura and associated with past exposure to asbestos fibres [1]. Although knowing the patient's mutational landscape does not yet significantly affect the therapeutic approach in PM, in the

☆ Twitter handle: Pleural fluids are an essential complement to solid biopsies for the accurate mutational characterization of pleural mesothelioma.

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future it could be pivotal for defining dominant clones and identifying potentially targetable or resistant sub-clones [2]. To date, the molecular characterization of PM relies mainly on solid tissue biopsies (STBs) collected at thoracoscopy. This invasive procedure provides only a partial picture of the intra-patient heterogeneity and does not allow capturing the multiple subclones that characterize this malignancy [2]. A multi-sampling approach would improve this characterization, but it is not easily feasible in clinical practice [2]. A better molecular characterization could be obtained for the minority of patients who can undergo to surgery for whom resected specimens could be available. In any case, these patients are followed up mainly with radio-imaging techniques that do not provide information on the molecular evolution occurring during cancer progression.

Moreover, the benefits of surgery in multimodal treatment are currently debated, and the diagnosis often occurs when the surgical approach is no longer feasible, making the resected specimens rarely available for the molecular characterization of the tumour [1]. Liquid biopsies (LB) could help to overcome these limitations. LBs consist of blood, pleural fluids (PF), urine, and other biological fluids, and their collection is minimally invasive and can be easily repeated over time. Analyzing the tumour DNA recovered from LBs could allow the evaluation of the patient's mutational status that in the future could be a crucial step for identifying potentially druggable mutations. Although no actionable modifications are currently known in PM, this strategy is already under intensive investigation for other malignancies, such as Non-Small-Cell Lung Cancer (NSCLC) [3]. Indeed, in NSCLC, a follow-up based on LBs can guide the choice of alternative tyrosine kinase inhibitors upon the appearance of novel tumour subclones characterized by specific *EGFR* mutations [3]. Thus, DNA from LBs could represent a valuable source of non-invasive biomarkers for personalized medicine. However, the usefulness of LB-derived DNA for the molecular characterization of PM has been poorly assessed. To the best of our knowledge, only one study has used PFs to evaluate the mutational landscape in PM so far [4]. Nevertheless, in that study, STBs were unavailable, preventing a direct comparison between STB and PFs as sources of tumour DNA for capturing the mutational heterogeneity that characterizes this malignancy.

To shed light on this aspect, we carried out the WES of the DNA extracted from STBs and their matched PFs of three patients. Then, we restricted the analysis to the genes most frequently mutated in PM, and we compared the single nucleotide variants (SNVs) detected in STBs and PF-derived samples of the same patient.

## 2. Materials and Methods

### 2.1. Patients and samples collection

The patients were recruited at the University Hospital of Cisanello (Pisa) between December 2020 and February 2021, with the approval of the ethics committee of the Azienda Ospedaliero Universitaria Pisana (dated February 7, 2013, protocol number 192/53) and following their informed consent. Patients were two 79-year-old males (C001 and C008), and one 77-year-old female (C002) who underwent thoracoscopy for diagnostic purposes and were then diagnosed with PM (all with the epitheliod histological subtype). For ethical reasons, we analysed only a tiny fraction of one of the several available withdrawals that were all sent to the pathological anatomy department for the classical routine diagnosis. Blood samples, PF, and STB were collected for each patient at the thoracoscopy. The blood samples were collected in EDTA tubes and processed through centrifugation ($400\times g$ for 30 min); the buffy coat (BC) was recovered and stored at $-80$ °C. PFs were centrifuged at $400\times g$ for 15 min to separate the supernatant (SRN) from the cell pellet (CP); the two components were individually stored at $-80$ °C. The STBs were directly stored at $-80$ °C without any further processing step, and their sizes were about 1 $mm^3$.

### 2.2. DNA extraction

DNA was extracted from BC and STB using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific; Waltham, MA, USA) and from PF (both SRN and CP) using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Velno, Netherlands), according to manufacturer's instructions. DNA concentration was quantified using the Qbit3 (Thermo Fischer Scientific; Waltham, MA, USA).

### 2.3. Next generation sequencing, filtering, and data analysis

WES was carried out on BC, STB, and PF (both SRN and CP) using a NextSeq 550 (Illumina; San Diego, CA, USA). The libraries for the sequencing of BC and STB were prepared using the Nextera DNA Flex Pre-Enrichment Library Prep and Enrichment (Illumina) and the sequencing indexes provided by the same manufacturer. For the PF-derived samples, the libraries were prepared with the TruSeq DNA Nano kit (Illumina), and the enrichment was done using the TruSight Rapid Capture (Illumina). The sequencing files for all sample types were aligned on the reference genome using the Burrows-Wheeler Aligner software [5], while Mutec2 [6] was employed for variant calling. Among the obtained SNVs, we considered only those within coding regions and with the following characteristics: **(i)** a minor allele frequency (MAF) $< 1$ % among Europeans (according to genomAD); **(ii)** a total depth (TD; defined as the number of reads covering that specific genomic location) $> 20\times$; **(iii)** an alternative/total reads ratio $<0.5$.

## 3. Results

Sequencing of STB and BC samples generated an average of 81.1 million reads per sample, with a mean length of 100 bases. On average, 93.5 % of the sequences aligned correctly with the reference genome. The final coverage within the targeted exome regions was 73.6×. The average number of reads for the CP samples was 94.2 million, with a mean length of 100 bases and 95.4 % of the reads

**Table 1**
List of single nucleotide variants for each patient in three sample types.

| | C001 | | | | | | STB | | SRN | | CP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| # | Consequence | Gene | TCGA Frequency (%) | codon | AA | ID | TD | AD | TD | AD | TD | AD | STB | SRN | CP |
| 1 | synonymous_variant | *TTN* | 13.75 | agC/agT | S | rs374346637, COSV59865090 | 256 | 38 | 348 | 135 | 372 | 14 | X | X | X |
| 2 | synonymous_variant | *OBSCN* | 3.75 | gcA/gcC | A | NM_001386125.1:c.21654A > C | | | 84 | 18 | 178 | 28 | | X | X |
| 3 | missense_variant | *TTN* | 13.75 | Ccc/Acc | P/T | COSV59952503 | | | 103 | 29 | | | | X | |
| 4 | missense_variant | *BAP1* | 27.5 | Cca/Aca | P/T | NM_004656.4:c.1159C > A | | | | | 522 | 16 | | | X |
| 5 | missense_variant | *NF2* | 23.75 | Gtc/Ttc | V/F | COSV58522820, COSV58529035 | | | | | 594 | 30 | | | X |
| 6 | missense_variant | *TTN* | 13.75 | aaC/aaA | N/K | COSV60225360 | | | | | 431 | 41 | | | X |
| 7 | stop_gained | *FAT4* | 7.5 | Gag/Tag | E/* | NM_001291303.3:c.1780G > T | | | | | 466 | 22 | | | X |

| | C002 | | | | | | STB | | SRN | | CP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| # | Consequence | Gene | TCGA Frequency (%) | codon | AA | ID | TD | AD | TD | AD | TD | AD | STB | SRN | CP |
| 8 | missense_variant | *TTN* | 13.75 | Gtg/Atg | V/M | rs771073631 | 90 | 31 | | | 92 | 38 | X | | X |
| 9 | frameshift_variant | *SETD2* | 10 | aaAGAAAca/aaca | KET/NX | NM_014159.7:c.2778_2782del | 111 | 35 | | | | | X | | |
| 10 | missense_variant | *OBSCN* | 3.75 | gTg/gGg | V/G | NM_001386125.1:c.1382T > G | | | 110 | 54 | 446 | 21 | | X | X |
| 11 | frameshift_variant | *TTN* | 13.75 | tTa/ta | L/X | NM_133379.5:c.12482del | | | 274 | 46 | | | | X | |
| 12 | synonymous_variant | *PTCH1* | 5 | ggC/ggA | G | COSV59471916 | | | 96 | 50 | | | | X | |
| 13 | synonymous_variant | *NF2* | 23.75 | acC/acT | T | rs769454739,COSV100098856, COSV104643314 | | | | | 279 | 14 | | | X |
| 14 | missense_variant | *TTN* | 13.75 | cCg/cAg | P/Q | COSV59893506,COSV60274263 | | | | | 301 | 44 | | | X |
| 15 | missense_variant | *TTN* | 13.75 | Gct/Act | A/T | rs72647885,COSV100630370 | | | | | 286 | 49 | | | X |
| 16 | missense_variant | *LATS2* | 10 | Ctg/Atg | L/M | rs1254526831 | | | | | 224 | 38 | | | X |
| 17 | stop_gained | *PTCH1* | 5 | tCa/tGa | S/* | CM070247,CM152924,COSV59487196 | | | | | 312 | 18 | | | X |

| | C008 | | | | | | STB | | SRN | | CP | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| # | Consequence | Gene | TCGA Frequency (%) | codon | AA | ID | TD | AD | TD | AD | TD | AD | STB | SRN | CP |
| 18 | missense_variant | *TTN* | 13.75 | Acc/Ccc | T/P | COSV60356392 | 236 | 17 | | | | | X | | |
| 19 | frameshift_variant | *SETD2* | 10 | Gaa/aa | E/X | NM_014159.7:c.2242del | 107 | 31 | | | | | X | | |
| 20 | synonymous_variant | *MUC16* | 3.75 | agT/agC | S | rs201630633,COSV66693897 | | | 34 | 34 | 202 | 44 | | X | X |
| 21 | synonymous_variant | *BAP1* | 27.5 | ctG/ctT | L | COSV56236553 | | | | | 571 | 18 | | | X |
| 22 | synonymous_variant | *BAP1* | 27.5 | ccC/ccA | P | COSV56233246 | | | | | 588 | 18 | | | X |
| 23 | missense_variant | *NF2* | 23.75 | Gct/Act | A/T | rs780430071,CD115314 | | | | | 602 | 28 | | | X |
| 24 | stop_gained | *TTN* | 13.75 | tCa/tAa | S/* | COSV59965477,COSV60280617 | | | | | 396 | 16 | | | X |
| 25 | synonymous_variant | *FAT4* | 7.5 | ctG/ctT | L | NM_001291303.3:c.1452G > T | | | | | 300 | 32 | | | X |
| 26 | missense_variant | *FAT4* | 7.5 | Ggt/Tgt | G/C | NM_001291303.3:c.10021G > T | | | | | 330 | 18 | | | X |
| 27 | missense_variant | *PTCH1* | 5 | Agc/Cgc | S/R | rs1564008885,COSV59494466 | | | | | 372 | 26 | | | X |

aligned to the reference genome. In the targeted exome regions, the resulting coverage value was 118×. Concerning the SRN samples, patient C008 resulted in only 3.5 million reads and an exome coverage of 4.5× because of the low amount of the recovered PF, while, for the other patients, the sequencing yielded 64.8 million reads and an exome coverage of 81.6×. Genomic positions with a TD < 20× were excluded. Firstly, following the exclusion of the germline variants by using BC samples, we assessed the agreement between STB, CP, and SRN samples within the same patient. In patient C001, 65 SNVs (6 % of the total SNVs found in the STB sample) were shared among STB, CP, and SRN samples. For C002 and C008, these common SNVs were 29 (3 % of STB) and 8 (0.6 % of STB), respectively. For patient C001, 20 % of the total SNVs detected in the STB were also found in CP (but not SRN) samples. For patients C002 and C008, these percentages were 20 and 25, respectively. Similarly, the SNVs found in SRN samples (but not in CP) were 9.5 % (C001), 4.9 % (C002), and 0.7 % (C008) of the total SNVs detected in STB samples. Next, we analysed the genes most frequently mutated in PM patients (>3 %), according to The Cancer Genome Atlas (https://www.cancer.gov/tcga), being highly likely candidate cancer driver genes (CCDG) for PM. Table 1 describes the variants found in the three patients and the correspondences among STB, CP, and SRN samples. Overall, patient C001 showed 7 SNVs in 5 genes. Of these SNVs, 1 within *TTN* was detected in the STB, SRN, and CP samples. C002 showed 10 SNVs in 6 genes. Of these SNVs, 2 were detected in the STB sample, and, of them, 1 (within *SETD2*) was exclusive of STB, while 1 (within *TTN*) was in common with CP. Lastly, C008 showed 10 SNVs in 7 genes. Of these SNVs, 2 (within *TTN* and *SETD2*) were detected in the STB sample and not in CP or SRN samples. As can be observed in Fig. 1 and Table 1, most of the SNVs were detected in SRN and/or CP but not in STB samples. This occurred for 6 out of 7 in patient C001 and 8 out of 10 in patients C002 and C008. Finally, each patient showed only 1 mutation in common between SRN and CP samples, within *OBSCN* in patients C001 (at position 2165) and C002 (at position 1382) and within *MUC16* in patient C008.

Among the CCDG, *TTN* and *NF2* harboured mutations in all patients. However, SNVs within *NF2* were detected only in CP samples. SNVs within *BAP1*, *FAT4, LATS2, MUC16, and PTCH1* were not detected in STB samples. Mutations of *BAP1* and *FAT4* were found only in CP samples of patients C001 and C008, and *LATS2* was found mutated only in the CP sample of patient C002. Mutations within *MUC16* were detected in patient C008 in both SRN and CP samples. For *PTCH1*, 2 SNVs were detected alternatively in SRN or CP samples from patient C002, whereas 1 SNV was found in the CP sample of patient C008. Among these genes, only *SETD2* was found mutated in STB samples (in patients C002 and C008) but not in CP or SRN samples. Overall, 9 mutations were found within *TTN*: 1 was detected in SRN, STB, and CP samples (C001), 1 in both STB and CP samples (C002), 1 in STB-only (C008), 2 in SRN-only (C001, C002), and 4 in CP-only samples (C001 C002, C008).

## 4. Discussion and conclusions

This work reinforces previous observations [7] and adds additional pieces of evidence showing that PM releases tumour-derived DNA and malignant cells into the PFs [4,8,9]. The analysis of PFs for molecular characterization of this cancer has been poorly

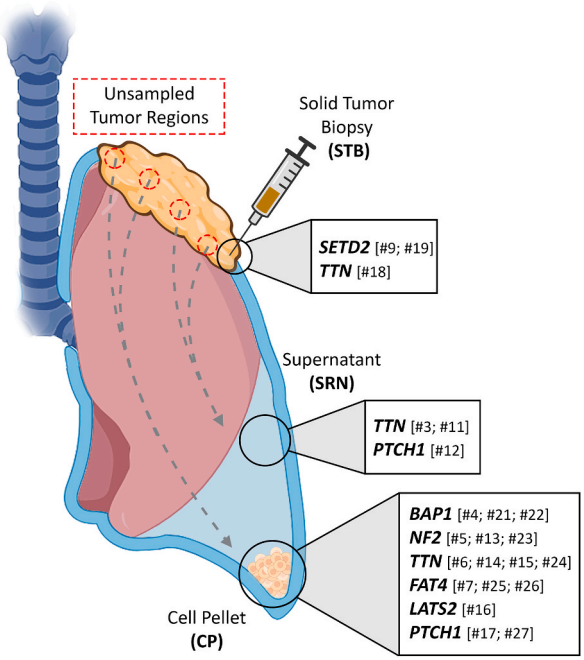


**Fig. 1.** Figure showing which genes, among those most frequently mutated in mesothelioma, were found mutated exclusively in the solid tissue biopsies or in the pleural fluids-derived samples (supernatant or cell pellet). The numbers between brackets identified specific mutations as reported in Table 1. Unsampled regions of the solid tumor (red dashed circles) are thought to be the source of the mutations found only in the supernatant or in the cell pellet. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

investigated. Seldomly, PFs have been studied to evaluate genotoxic damages [8] and DNA integrity index [9] in an attempt to detect novel biomarkers for PM. In this work, we analyzed the somatic SNVs detected in STB, SRN, and CP samples of 3 p.m. patients. It is important to stress that the SNVs that we identified (either in STBs or PFs) do not necessarily represent clinically relevant variants or subclones, as the aim of this study was just to understand whether PFs could provide a more detailed molecular landscape of the tumour than their STBs counterparts. For these reasons, we also included the synonymous SNVs in the analysis.

To our knowledge, only one study exploited PFs to assess the mutational landscape of PM patients [4]. In that study, the authors carried out a WES on DNA extracted from circulating cells retrieved in PFs and found the typical PM mutations affecting *BAP1*, *NF2*, *CDKN2A*, *TRAF7*, *LATS2*, and *SETD2* [4]. Among our patients, we found that most of the mutations affecting the typical PM-driver genes, including *BAP1*, *FAT4*, *LATS2*, *MUC16*, and *PTCH1,* were detectable exclusively in PF-derived samples, suggesting that analyzing only the STBs would have prevented the detection of these variants. Copy number loss and point mutations affecting *BAP1* are often considered the early main driver events of PM and, thus, are supposed to be present in every sub-clonal population. The observation that we detected *BAP1* mutations only in the PF but not in the STBs may raise some concerns about the tumor sampling during the biotic procedure, especially in the absence of a microscopic investigation. We must consider, however, that two of the three SNVs detected within *BAP1* were synonymous mutations and only the missense variant detected on patient C001 was likely to have deleterious effects (according to CADD). Overall, these considerations seem to suggest that these SNVs were probably passenger mutations and that they were not detected in the STBs due to the tumor heterogeneity. In this respect, different studies showed similar results, suggesting that the analysis of a single portion of the tumor may prevent the detection of the typical PM genetic alterations. For example, Wu et al. [10] reported that the common *CDKN2A* deletion was detectable at different degrees, with cells from the same tumor showing no detectable loss, hemizygous losses, and homozygous losses, highlighting a wide intra-tumor heterogeneity. Similar results were also obtained by Kiyotani et al. [11] by carrying out a WES on the DNA extracted from three sites of resected tumors (anterior, posterior, and diaphragm). A non-synonymous mutation of *BAP1* was found only in one of the six analysed patients. While this mutation was present in each of the three sample sites, its frequency varied a lot, ranging from 0.13 to 0.55 depending on the sapling site. Even more interestingly, they found that some non-synonymous mutations in other genes were detectable only in the DNA derived from one or two sampling sites, suggesting that a single-site sampling would have prevented their detection. Finally, it should be also noted that cancer databases (such as TCGA https://www.cancer.gov/tcga) reveal that most PM patients actually present loss of one copy of *BAP1*, therefore it could be possible that the patients herewith studied had such an alteration that could not be detected with our methods of analysis.

Notably, Sneddon et al. reported that the analysis of DNA derived from circulating PF cells allowed identifying mutations within genes that are not usually associated with PM, including *FGFR3*, *MUC4*, *HUWE1*, *GRM8*, *RIF1*, *SLCO5A1*, and *PCF11* [4]. Interestingly, we also found that *HUWE1* was mutated in all patients of our cohort, but the mutations were detectable only in PF-derived samples. Similarly, we found mutations in *RIF1* and *PCF11* in both patients C001 and C002 and *FGFR3* in patient C001 but, again, only in PF-derived samples.

Overall, our results support the hypothesis that PFs samples could be more representative of the PM mutational landscape than STBs. A limitation of the study was related to the low number of bioptic specimens employed, which could be inadequate considering the wide and intrinsic genetic heterogeneity of this malignancy [2]. In fact, in order to not hamper the regular clinical diagnostic process, we investigated only a tiny fraction of one of the several specimens employed for histopathology, and, for ethical reasons, we could not obtain extra biopsies. Therefore, chances are that the results of STBs could be limited and not disclosing the full variability of the tumoral mass. Indeed, this further stresses the results of our work, since we are suggesting that LBs could be a valid implementation for extracting information not completely retrievable from normal STBs.

On the other hand, present results flaw the notion that LBs could capture the whole intra-patient variability. In fact, there was a share of putative cancer-driver SNVs detected only in STBs. Among them, a missense SNV within *TTN* in patient C008 and 2 different frame-shift variants within *SETD2* in patients C002 and C008. Moreover, *MUC4* showed 2 SNVs exclusive of STB-sample of patients C001 and C002. Despite the fact that these SNVs seem to occur as minor events, they alert about the dynamics occurring when cells and tumour DNA are released into biological fluids and suggest that LBs could be an important complement, but not a replacement, of STBs.

## Ethics declarations

This study was reviewed and approved by the ethics committee of the Azienda Ospedaliero Universitaria Pisana with the approval number: 192/53 dated February 7, 2013.

All patients provided written informed consent to participate in the study and for their data to be published.

## Data aviability

The raw sequencing data of this project are available at: https://fpsonlus-my.sharepoint.com/:f:/g/personal/repository_data_fpsonlus_onmicrosoft_com/ErYdYnDieCVPlIkf74s7Oj0B7rPn_WfLWgGischOWvfr7g?e=Rn5XBo.

## CRediT authorship contribution statement

**Roberto Silvestri:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Filomena Rea:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Marianna Vitiello:** Writing – review & editing, Writing – original draft, Visualization, Data curation. **Gabriele Moretti:** Investigation, Formal analysis, Data curation, Conceptualization.

**Vittorio Aprile:** Resources. **Marco Lucchi:** Resources. **Paolo Aretini:** Formal analysis. **Chiara Maria Mazzanti:** Resources. **Stefano Landi:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Federica Gemignani:** Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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