Molecular Signatures Associated With the Development of Pulmonary Toxicities in Mesothelioma Patients Treated With Radical Hemithoracic Radiation Therapy

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36 **Abstract**

37 **Background.** Radical Hemithoracic Radiotherapy (RHR), after lung-sparing surgery, has recently become
38 a concrete therapeutic option for malignant pleural mesothelioma (MPM), an asbestos-related, highly
39 aggressive tumor with increasing incidence and poor prognosis. Although the toxicity associated to this
40 treatment has been reduced, it is still not negligible and must be considered when treating patients.
41 Genetic factors appear to play a role determining radiotherapy toxicity. The aim of this study is the
42 identification of genetic markers able to predict the relative susceptibility for newly diagnosed MPM
43 patients to develop lung adverse effects if they were to be treated with RHR.

44 **Methods.** The study included individuals with MPM, treated with lung-sparing surgery and neoadjuvant
45 chemotherapy, followed by RHR with curative intent, and followed up prospectively for development of
46 pulmonary toxicity. Due to the impact of grade 3 pulmonary toxicities on the quality of life, for further
47 genetic analyses patients were divided into a none or tolerable pulmonary toxicity group (Grade ≤ 2) and
48 a severe pulmonary toxicity group (Grade=3). Whole exome sequencing (WES) was performed to
49 identify genetic variants in biological pathways associated to pulmonary toxicity after radiotherapy.

50 **Results.** We identified crucial pathways driving the lung response to ionizing radiations in patients
51 affected by mesothelioma: by affecting both the fibrinolytic activity and RNA editing pathways,
52 irradiation could be responsible of the severe toxicity events reported by some patients, who present
53 specific mutations in genes involved in these pathways.

54 **Conclusions.** Our preliminary results could pave the way for the definition of a panel of predictive
55 genomic variants, capable of supporting the management of MPM patients. By allowing for early
56 identification of patients at high risk for treatment-dependent pulmonary toxicity, this predictive tool
57 could play a major role in the design of new therapeutic combinations.

58

59

60 **BACKGROUND**

61

62 Malignant pleural mesothelioma (MPM) is an asbestos-related, highly aggressive tumor derived from
63 cells of the pleura, with increasing incidence (expected to peak in the period 2020-2024) (1) and poor
64 prognosis. Such dismal outcome also derives from the complex management of the disease, given that
65 the scientific community still debates about the best protocol to follow, based on the three therapeutic
66 options that are currently available: surgery, radiotherapy and chemotherapy (2-4).

67 Until recently it was thought that the routine administration of radiotherapy in patients with MPM was
68 not supported by evidence (5). In recent years, modern radiation techniques have been applied post-
69 surgery, allowing for more effective sparing of normal tissue, thus enabling higher radiation doses at the
70 tumor site. Researchers from the National Cancer Institute of Aviano have published a series of
71 prospective studies (6-8) proving that radical intensity modulated radiation therapy, after lung-sparing
72 surgery, leads to excellent loco-regional control and survival in MPM patients. A median overall survival
73 (OS) of 25.6 months and a 2-year OS rate of 58% are among the best results observed in recent studies
74 (9-11), supporting the idea that this approach represents a concrete therapeutic option for MPM.

75 Although the toxicity associated to these treatments has been drastically reduced, it is still not negligible
76 and must be taken into consideration when treating patients (12). Pulmonary toxicities are very
77 common among mesothelioma patients undergoing radiotherapy on the entire hemithorax even if the
78 treatment is well-tolerated. Albeit infrequent, worse adverse effects, like grade 2 or 3 fibrosis and
79 pulmonary embolism, can be seen, and the management of these toxicities is still challenging.

80 Improved ways of predicting, prior to treatment, the risk of development of adverse lung effects after
81 radiotherapy may result in more promising personalized treatments and in a reduced incidence and
82 severity of late effects. There is increasing recognition that the cause of normal tissue toxicity is

83 multifactorial and includes genetic factors in addition to dosimetric parameters, patient age, smoking
84 history, concurrent treatments, and comorbidities (13-15).

85 In the last decade, more than one hundred papers have been published addressing possible associations
86 between genetic germline variants and risk of normal tissue toxicity after radiotherapy. With few
87 exceptions, however, these relied on relatively few studies that used a candidate gene approach [single
88 nucleotide polymorphisms (SNPs) analysis] (16), and the association results have not been replicated
89 (17-18).

90 Recently, genome-wide association analyses (GWAS), including meta-analysis, performed by the
91 Radiogenomics Consortium (RgC; epi.grants.cancer.gov/radiogenomics/) have identified several novel
92 SNPs within genes not previously linked to radiotherapy toxicity, in patients affected by different types
93 of cancers, such as breast, prostate and lung (19, 20). Nevertheless, genetic susceptibility to
94 radiotoxicity in nonsyndromic individuals remains poorly understood. To our knowledge, whole exome
95 analysis has not previously been considered as a potential tool to assess the toxicity of radiotherapy for
96 lung/pleural cancer, based on patients' genetic background. So, with the aim of contributing to the
97 identification of a possible role for genetic variations in the coding and their flanking intronic regions,
98 in the response to radiotherapy in MPM patients, we performed whole exome sequencing, coupled with
99 bioinformatic analysis, to identify not only the genetic variants associated to pulmonary toxicity after
100 radiotherapy, but also to unravel biological pathways that can better elucidate the observed
101 phenotypes.

102

103 **METHODS**

104 **Study Participants**

105 The study included individuals with MPM, treated with non-radical surgical treatment, such as partial
106 pleurectomy/decortication (P/D) or biopsy only, and neoadjuvant platinum-based plus pemetrexed

107 chemotherapy for MPM, followed by high dose radiation therapy with curative intent, and followed up
108 prospectively for development of (lung/pulmonary) toxicity. Other inclusion criteria were: age ≥ 18
109 years, proven gross residual disease after surgery, stage I-IVA (according to TNM stage 7th edition),
110 Eastern Cooperative Oncology Group (ECOG) performance status score 0-2, pulmonary function of at
111 least 50% of predicted, technical feasibility for delivery of Radical Hemithoracic Radiotherapy (RHR),
112 satisfactory bone marrow function (white blood cells $\geq 2000/\mu\text{L}$, platelet $\geq 100.000/\mu\text{L}$, haemoglobin $>$
113 10 g/dL). Exclusion criteria were: pathologic contralateral mediastinal nodes (N3), metastatic MPM
114 (Stage IVB), or intra-scissural disease. Tumour histology was classified as epithelioid and non-epithelioid
115 (sarcomatoid and biphasic). All patients were staged by lung and abdomen contrast-enhanced
116 computed tomography (CT) scans and a 18F-fluorodeoxyglucose (18FDG)-positron emission tomography
117 (PET)/CT.

118 Between August 2014 and May 2018, 49 patients who received Radical Hemithoracic Radiotherapy
119 (RHR) were included in this study. Patients were treated with helical IMRT, delivering the dose with
120 Accuray Tomotherapy System. The radiation therapy technique has previously been described in detail
121 (7, 8, 11). Patients received 50 Gy in 25 fractions (except for one case, later described), plus an eventual
122 boost to 60 Gy on PET positive areas.

123 OS was defined as the time (years) intercourse since randomization to death from any causes, or the last
124 follow-up (until December 2020, 1st), and estimated with the Kaplan-Meier method, p value was
125 calculated with Long-rank test.

126 This prospective study was conducted according to the ethical principles of the Declaration of Helsinki
127 and approved by the local Ethical Committee (*Comitato Etico Indipendente del CRO di Aviano*, CRO-
128 2013-38) and written informed consent was obtained from all the patients. The CRO-biobanking service
129 managed and stored all biological samples before use for the present project (authorization for analyses
130 obtained through protocol number 6825/D).

131 **Assessment of Radiotherapy Toxicity**

132 Toxicity was assessed using the Common Terminology Criteria for Adverse Events version 3.0, and
133 divided into acute toxicity (during treatment), early toxicity (1-6months from the end of RHR) and late
134 toxicity (>6months from the end of RHR).

135 Acute toxicity was assessed weekly during radiation. Following completion of radiation treatment,
136 patients were reviewed at 1, 3, 6 (early toxicity), 8 and 12 months in the first year (late toxicity) and
137 every 4 months from the start of second year or before for clinical need. All respiratory toxicities
138 outcomes were analyzed: cough, dyspnea, fibrosis, pneumonitis and pulmonary thromboembolic event.

139

140 **Genotyping, Quality Control and Imputation/ Statistical Analysis**

141 **DNA extraction**

142 Germline DNA from whole blood (blood samples collected in anticoagulant-citrate-dextrose before the
143 start of radiation treatment) was extracted from patients using the DNeasy Blood & Tissue Kit (Qiagen,
144 Milan, Italy) following the manufacturer's protocol. DNA quality and quantity were evaluated by agarose
145 gel (2%) electrophoresis and using the Invitrogen Qubit assay (Thermo Fisher Scientific, Milan, Italy). All
146 samples extracted successfully passed quality control, based on MacroGen ([https://dna.macrogen-](https://dna.macrogen-europe.com/eng/)
147 [europe.com/eng/](https://dna.macrogen-europe.com/eng/)) requirements for exome sequencing.

148 **Exome sequencing**

149 Exome sequencing was performed in outsourcing using the service provided by MacroGen Europe
150 (Amsterdam, The Netherlands). Briefly, the Exome Sequencing Analysis, aiming to 150x coverage, used
151 the Illumina® SureSelect Human V7 Kit Library preparation and sequencing reaction, in an Illumina®
152 HiSeq 2500 System, generating pair-end reads of 125 base pairs.

153 Illumina universal adapters were removed using Trim Galore 0.6.1
154 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Reads with length below 15 base

155 pairs and with low Phred score ($Q < 20$) were also removed using the same software. Quality control
156 before and after these procedures were evaluated by fastQC
157 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>).

158 After QC, the FASTQ file with the raw reads was aligned using Burrows-Wheeler Aligner Software
159 Package (21), specifically the bwa-mem tool, against the Reference Human Genome version 38
160 (GRCh.38). We then used Picard tools (<https://broadinstitute.github.io/picard/>) to mark duplicates, and
161 GATK V. 4.1.2.0 (<https://software.broadinstitute.org/gatk/>) for base recalibration. Strelka2 was used for
162 variant calling, when the software was employed in the exome mode of analysis (22). Finally, we used
163 GATK again for the exclusion of low-quality variants.

164 Variant annotation was performed using the ANNOVAR (23) software with databases relative to the
165 GRCh.38 reference genome (refGene, cytoBand, wgRna, 1000g_2015_aug_all, gnomad30_genome,
166 dbcsnv11, dbnsfp35a, clinvar_20200316 and avsnp151). R Software (24) was employed to manipulate
167 ANNOVAR results for a descriptive and inferential analysis.

168 We divided the analysis into two main parts: individual, which consists in summarizing the descriptive
169 data for each sequenced sample; and group data, which aims at drawing comparisons between patients
170 with severe toxicity versus no severe toxicity. In addition, for both individual and group categories, we
171 performed a 'Variant Enrichment Analysis'.

172 Principal component analysis (PCA) of variant distribution was accomplished using stats R packages in a
173 non-centred approach and scaled to have unit variance before the analysis (R Core Team 2020).

174 Variant Enrichment Analysis works similarly to Gene Enrichment Analysis for expression data: we
175 investigated if there were statistical differences between the numbers of mutations in a pathway
176 compared to a reference dataset. We used the R package 'ReactomePA' to obtain pathway information
177 on each of the genes containing at least one mutation in the individual dataset (25). Then, we obtained
178 variant data from GnomAD Exome 3.0 (26) to use as a reference dataset. It is important to mention that,

179 since the occurrence of some mutations may be related to specific populations, we considered for this
180 dataset only the Non-Finnish European (nfe) variant information.

181 Once we had the number of mutations per pathway in the individual dataset and in the reference
182 dataset, we used Fisher's Exact test with False discovery Rate (FDR) to identify statistical differences in
183 the number of variants. Adjusted p-values < 0.05 were considered significant in this analysis. For group
184 data, we used Venn diagrams to summarize "enriched" pathways exclusive for each group.

185 Human Phenotype Ontology (HPO) database was used to retrieve genes associated to the main severe
186 toxicity response in order to extract the patients' variations from the respective genes.

188 RESULTS

189 Patients

190 Forty-nine patients were referred for radiation treatment following non-radical surgical treatment (such
191 as partial P/D or biopsy only) between August 2014 and May 2018. Patients' characteristics are
192 summarized in Table 1. 48 patients completed treatment as prescribed. One patient, requiring a
193 treatment break due to anemia and trombocytopenia, globally received 32 Gy in 16 fractions. One
194 patient developed infective pneumonia independent of RT, and discontinued the treatment, completing
195 the prescribed course at a later moment. 45 patients received the simultaneous boost.

Table 1. Patient and tumor characteristics			
Characteristic		Value	%
Gender			
	Male	40	81.6
	Female	9	18.4
Age (years)			
	Median	69	NA
	Range	33-81	NA
Laterality			
	Right	26	53.0
	Left	21	42.9
	nd	2	4.1
Histological subtype (n)			
	Epithelioid	46	93.9
	Non epithelioid	3	6.1
Pathological stage			
	Stage I-II	20	40.8
	Stage III-IV	29	59.2

196 NA, not applicable

197

198 **Early, Acute and Late toxicity**

199 Reported adverse effects are summarized in Table 2, divided in events observed during treatment (early
200 toxicity), between 1 and 6 months after the end of RT (acute toxicity), and after 6 months of follow up
201 (late toxicity), and classified as non-pulmonary or pulmonary toxicities.

202 Globally, for acute and late toxicities the number of events in the non-pulmonary toxicity group tended
203 to diminish, except for the rise in liver gamma-glutamyltransferase (GGT), commonly observed in
204 patients treated on the right hemithorax (not shown). Fractures and pericardial effusions are typically
205 late events, and were observed in a small number of patients, 2 and 3 cases respectively. Conversely,
206 pulmonary toxicity events tended to develop after treatment and to last in time. One third of the
207 patients developed low-grade cough by 6 months after RT, or at a later follow up. Dyspnea was reported
208 in 37% of patients as acute toxicity, mainly low-grade, but became a severe toxicity in 8% of patients, as
209 a late event. Fibrosis developed in 3 patients (2 severe), and pneumonitis in 7 cases, 4 of which were
210 grade 3. Finally, 3 thromboembolic events were reported after treatment.

211 Due to the impact of grade 3 pulmonary toxicities on the quality of life, for further genetic analyses
212 patients were divided into two groups: no severeTox (NoSTox), none or tolerable toxicity (Grade 0 to 2)
213 ($\text{grade} \leq 2$) (N=40) and severeTox (STox) ($\text{grade} = 3$) (N=9) based on the development of at least one among
214 the following: cough, dyspnea, fibrosis, pneumonitis and/or thromboembolic event. STox patients'
215 characteristics and reported adverse effects are summarized in Table 3. No Grade 4 or 5 toxicities were
216 recorded during treatment.

217 Among the 49 patients included, median OS was 25 months, and 2-year OS rate was 51.4%. No
218 significative survival differences were observed between the NoSTox and the STox group.

219

Table 2. Non-pulmonary and pulmonary events observed as Acute, Early and Late toxicities			
Non-pulmonary Toxicity	Early Toxicity n (%)	Acute Toxicity n (%)	Late Toxicity n (%)
Anemia <ul style="list-style-type: none"> Grade 1-2 Grade 3 	0 (0) 1 (2)	0 (0) 0 (0)	1 (2) 0 (0)
Chest wall pain <ul style="list-style-type: none"> Grade 1-2 Grade 3 	12 (24) 1 (2)	12 (24) 0 (0)	5 (10) 1 (2)
Dermatitis <ul style="list-style-type: none"> Grade 1-2 Grade ≥3 	11 (22) 1 (2)	2 (4) 0 (0)	0(0) 0(0)
Fatigue <ul style="list-style-type: none"> Grade 1-2 Grade 3 	19 (39) 1 (2)	10 (20) 1 (2)	4 (8) 1 (2)
Fracture	0 (0)	0(0)	2 (4)
GGT <ul style="list-style-type: none"> Grade 1-2 Grade 3 	9 (18) 0 (0)	11 (22) 1 (2)	5 (10) 3 (6)
Nausea/Vomiting <ul style="list-style-type: none"> Grade 1-2 Grade 3 	23 (47) 0 (0)	1 (2) 0 (0)	1 (2) 0 (0)
Oesophagitis <ul style="list-style-type: none"> Grade 1-2 Grade 3 	34 (69) 2 (4)	6 (12) 0 (0)	1 (2) 0 (0)
Pericardial effusion <ul style="list-style-type: none"> Grade 1-2 Grade 3 	0 (0)	0(0) 1(2)	0 (0) 2 (4)
Pulmonary Toxicity			
Cough <ul style="list-style-type: none"> Grade 1-2 Grade 3 	5 (10) 0 (0)	16 (33) 0 (0)	14 (29) 0 (0)
Dyspnea <ul style="list-style-type: none"> Grade 1-2 Grade 3 	9 (18) 0 (0)	17 (35) 1 (2)	7 (14) 4 (8)
Fibrosis <ul style="list-style-type: none"> Grade 1-2 Grade 3 	N.A.	0(0) 1(2)	1 (2) 1 (2)
Pneumonitis <ul style="list-style-type: none"> Grade 1-2 Grade 3 	1 (2) 0 (0)	1 (2) 2 (4)	1 (2) 2 (4)
Thromboembolic event	0 (0)	2 (4)	1 (2)

221 GTT, gamma-glutamyltransferase.

222

223

Table 3. STox: patient, tumor and pulmonary toxicities characteristics									
Patient ID#	Gender	Age	Laterality	Histological subtype	Pathological stage		Early Toxicity	Acute Toxicity	Late Toxicity
					T stage	N stage			
2	M	71	Right	Epithelioid	4	0	none	Dyspnea G2, Pneumonitis G3, Cough G2, Thromboembolic eventG3	Dyspnea G3
12	M	63	Left	Epithelioid	2	0	Pneumonitis G1	Fibrosis G3	none
17	M	72	Left	Epithelioid	2	0	none	none	Thromboembolic event G3
20	F	76	Right	Epithelioid	3	0	none	none	Pneumonitis G3, Cough G2
53	M	65	Right	Epithelioid	2	0	none	none	Cough G2, Dyspnea G2, Pneumonitis G3
58	F	65	Left	Epithelioid	3	2	none	Dyspnea G3	Cough G2, Pneumonitis G3, Dyspnea G3,
61	F	72	Right	Epithelioid	2	0	none	Dyspnea G2	Cough G2, Dyspnea G3
70	M	78	Left	Epithelioid	3	0	Dyspnea G2	Pneumonitis G3, Thromboembolic event G3	none
94	M	75	Left	Epithelioid	3	0	Dyspnea G1	Dyspnea G2	Pneumonitis G2, Fibrosis G3

224

225 Genetic analysis

226 The average number of variants per sample in the NoSTox group was 174,564 with 35,101 (20.12% of
 227 the total) being specific exon variants (ExonVars). For the STox group, the average number of mutations
 228 per sample was 177,555 with 35,142 (19.79%) ExonVars. Subsequently, ExonVars were categorized in
 229 two groups: the first group included the so-called Functional Variants (FunctVar), comprising frameshift
 230 insertion/deletion, start gain/loss and stop loss variants. The second group comprised the Impact
 231 Variants (ImpactVar), which are the mutations predicted as ‘damaging’ by at least one of the softwares:
 232 SIFT, Polyphen2, LRT, Mutationtaster, Mutationassessor, FATHMM, Provean, Metasvm, Metalr and
 233 M_cap.
 234 For the NoSTox group, on average, we found 324 FunctVar and 4,022 ImpactVar, whereas, for the STox
 235 group, we identified 331 and 3,980 FunctVar and ImpactVar, respectively. We then filtered NoSTox and
 236 STox common variants in order to retrieve only the variants exclusive to the STox group; 43 FunctVar and
 237 338 ImpactVar were found (Supplementary Tables 1 - Datasheet 1 and 2).

238 At the gene level, , in the NoSTox group, the average number of genes bearing variants was 17,994 with
 239 12,466 (69.28%) presenting at least one ExonVar; in the STox group mutations were detected in 18,059
 240 genes, with 12,465 (69.02%) genes carrying at least one ExonVar. Two-hundred sixty-seven genes had at
 241 least one Functional mutation, in the NoSTox group, and 264 in the STox group. The number of genes
 242 with at least one Impact mutation was 2,807 and 2,775 for the NoSTox and STox groups, respectively.
 243 The number of exclusive genes carrying functional or impact mutation on the STox group were 33 and
 244 225, respectively (Supplementary Tables 1 – Datasheet 3 and 4).
 245 Based on the results obtained considering variations present only in the STox patients, we retrieved
 246 the pathways disrupted by the identified mutations, without, however, detecting a significant specific
 247 pathway involved in the clinical phenotypes observed (data not shown).
 248 Furthermore, with the aim of applying a wider approach to pathway analysis, we performed a Variant
 249 Enrichment Analysis based on Functional and Impact mutations; 47 pathways showed a significantly
 250 increased number of Functional mutations exclusively in the NoSTox group (Table 4). For the STox
 251 group, we observed four exclusive pathways (Table 5). Regarding Impact mutations, we observed 19 and
 252 3 exclusive pathways for NoSTox and STox group, respectively (Tables 6 and 7).
 253 Tracing back the variants possibly involved in those pathways, we noticed 243 functional variants (along
 254 64 genes), for the **NoSTox** group, and 9 variants in for the **STox** (in 5 genes) (supplementary tables 2 and
 255 3). Considering the pathways with impact mutations, there are 2,908 variants (in 645 genes) present in
 256 the pathways exclusive of the NoSTox group, while only one mutation (one gene) is present in the
 257 pathways of the STox group (Supplementary tables 4 and 5).
 258 Despite the genetic differences found between NoSTox and STox, principal component analysis (PCA)
 259 showed a homogeneous distribution of variants in both groups. Thus, no specific genetic signature was
 260 observed in NoSTox and STox (Figure 1). However, in all analyses, patient 53 showed relevant significant

261 genetic distance compared to all other patients regardless of whether they were NoSTox or STox,
262 suggesting the presence of a specific genetic signature.

263

264
265

266 **Figure 1.** Principal component analysis (PCA) of genetic variants distribution in the NoSTox (red x) and
267 STox (blue x) groups. The variant distribution is homogeneous, no differences between the NoSTox and
268 STox groups can be observed. Patient 53# (red square) from the STox group shows ramarkable genetic
269 distance from all other patients, regardless of whether they were NoSTox or STox, thus suggesting the
270 presence of a specific genetic signature for this individual that is completely independent of the studied
271 phenotype.

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Table 4. List of the Reactome Pathways retrieved from Variant Enrichment Analysis (VEA) for functional mutation and the patients that they are found in the NoSTox group.

DB_ID	Pathway Name	Patient ID#	Number of Patients
R-HSA-73776	RNA Polymerase II Promoter Escape	x7,x22,x55	3
R-HSA-73779	RNA Polymerase II Transcription Pre-Initiation And Promoter Opening	x7,x22,x55	3
R-HSA-75953	RNA Polymerase II Transcription Initiation	x7,x22,x55	3
R-HSA-76042	RNA Polymerase II Transcription Initiation And Promoter Clearance	x7,x22,x55	3
R-HSA-72200	mRNA Editing: C to U Conversion	x9,x49,x29	3
R-HSA-75072	mRNA Editing	x9,x49,x29	3
R-HSA-75094	Formation of the Editosome	x9,x49,x29	3
R-HSA-73780	RNA Polymerase III Chain Elongation	x10	1
R-HSA-73980	RNA Polymerase III Transcription Termination	x10	1
R-HSA-74158	RNA Polymerase III Transcription	x10,x32	2
R-HSA-749476	RNA Polymerase III Abortive And Retractive Initiation	x10,x32	2
R-HSA-76046	RNA Polymerase III Transcription Initiation	x10,x32	2
R-HSA-76061	RNA Polymerase III Transcription Initiation From Type 1 Promoter	x10,x32	2
R-HSA-76066	RNA Polymerase III Transcription Initiation From Type 2 Promoter	x10,x32	2
R-HSA-76071	RNA Polymerase III Transcription Initiation From Type 3 Promoter	x10,x32	2
R-HSA-8850843	Phosphate bond hydrolysis by NTPDase proteins	x14	1
R-HSA-75105	Fatty acyl-CoA biosynthesis	x15	1
R-HSA-73728	RNA Polymerase I Promoter Opening	x21,x39,x43	3
R-HSA-74749	Signal attenuation	x21,x39,x43	3
R-HSA-74751	Insulin receptor signalling cascade	x21,x39,x43,x16	4
R-HSA-879415	Advanced glycosylation endproduct receptor signalling	x21,x39,x43	3
R-HSA-881907	Gastrin-CREB signalling pathway via PKC and MAPK	x21,x39,x43	3
R-HSA-8853659	RET signaling	x21,x50	2
R-HSA-77075	RNA Pol II CTD phosphorylation and interaction with CE	x22,x55	2
R-HSA-879518	Transport of organic anions	x43,x48	2
R-HSA-8852405	Signaling by MST1	x43,x48,x79	3
R-HSA-73942	DNA Damage Reversal	x62,x82,x85	3
R-HSA-73943	Reversal of alkylation damage by DNA dioxygenases	x62,x82,x85	3
R-HSA-75109	Triglyceride biosynthesis	x62,x77,x96	3
R-HSA-8848021	Signaling by PTK6	x62,x74	2
R-HSA-8849474	PTK6 Activates STAT3	x62	1
R-HSA-5357956	TNFR1-induced NFkappaB signaling pathway	x74	1
R-HSA-5358346	Hedgehog ligand biogenesis	x74	1
R-HSA-5362768	Hh mutants that don't undergo autocatalytic processing are degraded by ERAD	x74	1
R-HSA-73893	DNA Damage Bypass	x74,x37	2
R-HSA-75815	Ubiquitin-dependent degradation of Cyclin D	x74	1
R-HSA-8849469	PTK6 Regulates RTKs and Their Effectors AKT1 and DOK1	x74	1
R-HSA-8852276	The role of GTSE1 in G2/M progression after G2 checkpoint	x74,x29	2
R-HSA-8854050	FBXL7 down-regulates AURKA during mitotic entry and in early mitosis	x74	1

R-HSA-75035	Chk1/Chk2(Cds1) mediated inactivation of Cyclin B:Cdk1 complex	x84	1
R-HSA-73614	Pyrimidine salvage	x87	1
R-HSA-804914	Transport of fatty acids	x87	1
R-HSA-73843	5-Phosphoribose 1-diphosphate biosynthesis	x92,x37,x69	3
R-HSA-844615	The AIM2 inflammasome	x16	1
R-HSA-8862803	Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models	x56	1
R-HSA-72764	Eukaryotic Translation Termination	x88	1
R-HSA-5358493	Synthesis of diphthamide-EEF2	x96	1

276

Table 5. List of the Reactome Pathways retrived from Variant Enrichement Analysis (VEA) for functional mutation in the STox group.

DB ID	Pathway Name	Patient ID#	Number of Patients
R-HSA-75205	Clot	x2	1
R-HSA-72649	Translation initiation complex formation	x17,x53	2
R-HSA-72695	Formation of the ternary complex, and subsequently, the 43S complex	x53	1
R-HSA-72702	Ribosomal scanning and start codon recognition	x53	1

277

Table 6. List of the Reactome Pathways retrived from Variant Enrichement Analysis (VEA) for impact mutation in the NoSTox group.

DB ID	Pathway Name	Patient ID#	Number of Patients
R-HSA-75035	Chk1/Chk2(Cds1) mediated inactivation of Cyclin B:Cdk1 complex	x14,x15,x19,x31,x43,x48,x84,x85,x87,x37,x50	11
R-HSA-8849469	PTK6 Regulates RTKs and Their Effectors AKT1 and DOK1	x14,x62,x55,x88	4
R-HSA-202403	TCR signaling	x31	1
R-HSA-202424	Downstream TCR signaling	x31,x16	2
R-HSA-202427	Phosphorylation of CD3 and TCR zeta chains	x31,x16	2
R-HSA-202430	Translocation of ZAP-70 to Immunological synapse	x31,x85,x16	3
R-HSA-202433	Generation of second messenger molecules	x31,x16	2
R-HSA-388841	Costimulation by the CD28 family	x31	1
R-HSA-389948	PD-1 signaling	x31,x16	2
R-HSA-8853336	Signaling by plasma membrane FGFR1 fusions	x39,x52,x56	3
R-HSA-8849474	PTK6 Activates STAT3	x62,x55	2
R-HSA-198933	Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	x74	1
R-HSA-6805567	Keratinization	x84	1
R-HSA-844615	The AIM2 inflammasome	x84	1
R-HSA-75896	Plasmalogen biosynthesis	x50,x66,x88	3
R-HSA-8849468	PTK6 Regulates Proteins Involved in RNA Processing	x55,x59,x88	3
R-HSA-977347	Serine biosynthesis	x71	1
R-HSA-381753	Olfactory Signaling Pathway	x96	1
R-HSA-8853333	Signaling by FGFR2 fusions	x96	1

278

Table 7. List of the Reactome Pathways retrived from Variant Enrichement Analysis (VEA) for impact mutation in the STox group.			
DB ID	Pathway Name	Patient ID#	Number of Patients
R-HSA-75064	mRNA Editing: A to I Conversion	x94	1
R-HSA-75102	C6 deamination of adenosine	x94	1
R-HSA-77042	Formation of editosomes by ADAR proteins	x94	1

279

280 **Genetics and Phenotype of the non severe toxicity group: Variant Enrichment Analysis**

281 The pathways identified in the NoSTox group are mainly involved in common and non-specific biological
 282 processes such as RNA processing, cell signaling, cell cycle and lipid synthesis. However, four pathways,
 283 related to inflammatory and fibrotic processes, were also found to be compromised, in silico (Table 4).

284 1) The first is the R-HSA-5357956 (TNFR1-induced NFkappaB signaling pathway). One functional
 285 mutation in this pathway was found in patient #74: a frameshift insertion in the Ubiquitin C
 286 (UBC) gene (ENTREZID: 7316; exon 2: c.2051dupG or p.V685Cfs*7; without rs ID; located on
 287 chr12 at 124911720 position) in heterozygosis.

288 2) The second is the R-HSA-844615 (The AIM2 inflammasome) a single functional mutation related
 289 to this pathway was observed in patient #16: a frameshift deletion in the Absent in melanoma 2
 290 (AIM2) gene (ENTREZID: 9447; exon 5: c.712delA or p.T238Hfs*14; without rs ID; located on
 291 chr1 at 159062697 position) (Table 4). Notably, this pathway was also found among the list of
 292 impact reactome pathways in a different patient who did not develop severe pulmonary
 293 toxicities (#84, Table 6). In this case, patient #84 bore a mutation on exon2 (c.C278G) of PYD And
 294 CARD Domain Containing (PYCARD) (ENTREZ ID: 29108; mutation without rs ID located on chr 16
 295 at position 31202200).

296 3) The third pathway identified as impaired in the NoSTox group, is the Insulin receptor signalling
 297 cascade pathway (R-HSA-74751). Three frameshift and one stopgain mutation were found in
 298 patients #21, #39, #43 and #16, respectively (Table 4). All frameshift mutations were located in
 299 the Mitogen-Activated Protein Kinase 3 (MAPK3) gene (ENTREZ ID: 5595). Patients #39 and #43

300 carried the mutation on exon1 c.37_38insC (without rs ID, located on chr16 at position
301 30123172). Patient #21 carried one mutation on exon c.38_39insC (without rs ID; located on
302 chr16 at position 30123171). The stopgain mutation of Patient #16 was located on exon1 of the
303 Phosphodiesterase 3B (PDE3B) gene (ENTREZ ID: 5140; c.C447A; whitout rs ID; located chr11 at
304 14644522 position).

305 4) The last pathway predicted to be impaired in the NSTox group is related to Plasmalogen
306 biosynthesis (R-HSA-75896), in patients #50, #66 and #88 (Table 6). The gene
307 Glyceronephosphate O-Acyltransferase (GNPAT) (ENTREZ ID: 8443) was mutated in patients #50
308 and #66, rs11122266 and rs767514222, respectively, both in exon 9. Patient #88 carried the
309 rs764286061 SNP in the Alkylglycerone Phosphate Synthase (AGPS) gene (ENTREZ ID: 8540).

310

311 **Genetics and Phenotype of the severe toxicity group: HPO and Variant Enrichment Analysis**

312 Using the HPO database, we selected 8 phenotypes: Pulmonary fibrosis (HP:0002206),
313 thromboembolism (HP:0001907), pneumonia (HP:0002090), recurrent streptococcus pneumoniae
314 infections (HP:0005366), interstitial pneumonitis (HP:0006515), bronchiolitis obliterans organizing
315 pneumonia (HP:0011945), acute infectious pneumonia (HP:0011949) and usual interstitial pneumonia
316 (HP:0031950). From these, 242 genes were filtered from the STox pulmonar grade 3 phenotypes:
317 fibrosis (67), thromboembolism events (25) and pneumonia (128) (Supplementary table 6).

318 Patients #12 and #94 presented fibrosis; Patients #2, #17 and #70 had thromboembolic events, and
319 patients #2, #20, #58 and #70 were diagnosed with pneumonitis.

320 Twenty-five Impact Variants, along 12 genes related to fibrotic phenotypes, were found in patients #12
321 and #94, compared to the other patients.

Two ImpactVar, along 2 genes, were associated with pneumonia in patient #2, #20, #58 and #70, compared to the other patients. Both mutations were characterized by a Combined Annotation Dependent Depletion (CADD)_Score predicting low impact on the protein.

Four Impact Variants in the myopalladin gene (MYPN) (ENTREZ ID: 84665) were associated to thromboembolic event in patient #2, #17 and #70. This gene has been associated with cardiomyopathy, mainly dilated cardiomyopathy (27). However, no direct associations with lung thromboembolism have been described yet, so it is probable that this gene has bears no relation, at least not a direct one, with the observed adverse events.

Regarding to the STox Enrichment Variant Analysis, two pathways, identified in silico, achieved potential to translate the severe toxicity phenotype.

1) A single stopgain mutation on the Serpin Family F Member 2 (SERPINF2) gene (rs374446894), involved in the Dissolution of Fibrin Clot pathway (R-HSA-75205), was altered in patient #2, who developed a pulmonary thromboembolic event (Table 5).

2) A high impact non-synonymous mutation (vi1.154602065, CAAD 23, Impact Score 7/10) on the Adenosine Deaminase RNA Specific (ADAR) gene, involved in Formation of editosomes by ADAR proteins (R-HSA-77042), was carried by patient #94, who progressed to a high grade of fibrosis (Table 7).

DISCUSSION

The employment of Radical Hemithoracic Radiotherapy in MPM has shown to confer a survival advantage but is associated with a non negligible toxicity profile in a fraction of patients (8, 11). This treatment should be offered to most mesothelioma affected patients as it increases 2-year survival in inoperable patients to 58%, compared to the 28% rate reached by those who receive only CT or palliative radiotherapy (11). However, the toxicities associated with the treatment can have a relevant

346 impact on the quality of life, at least in a fraction of patients. Thus, the early identification of patients
347 who are at higher risk of developing severe toxicities could allow for better selection of patients eligible
348 for radical radiotherapy with limited side effects.

349 Although the cause of normal tissue toxicity is multifactorial (13-15), genetic factors could play a
350 significant role in determining radiation response (28).

351 The molecular events that lead to normal tissue injury are complex and span a variety of biologic
352 processes, including oxidative stress, apoptosis, inflammation and release of proinflammatory and
353 profibrogenic cytokines (29).

354 Pulmonary toxicity can heavily affect patient quality of life, causing mainly cough and dyspnea that last
355 over time. In grade 1-2 dyspnea there is a gradual worsening like exertional breathlessness, while grade
356 3 toxicity has an impact also on everyday life activities. Similarly, grade 1-2 pneumonitis is often
357 paucisymptomatic affecting only mildly the quality of life, while grade 3 pneumonitis can have a
358 permanent impact if it evolves in severe fibrosis (30), as observed in a few of the cases in the present
359 study. The inflammatory environment induced by RT, worsened by pneumonitis and subsequent
360 eventual fibrosis, could induce the development of fibrin clots, potentially leading to a thromboembolic
361 event (31), a complication which occurred in 3 of our patients, as acute and late toxic effect. This
362 particular aspect, however, needs to be further investigated given that literature evidence is still scanty,
363 and no predictive biomarkers for increased risk of pulmonary embolism are currently available.

364 The precise mechanisms underlying RP pathogenesis are unknown. They may involve radiation damage
365 of the alveolar epithelium and capillary endothelium, or an innate immune response to tissue
366 inflammation and injury (32-35). Radiation-induced pulmonary fibrosis, characterized by inflammatory
367 cell infiltration, fibroblast proliferation, and excessive deposition of extracellular matrix (ECM) proteins,
368 such as collagen, in the lung parenchyma (36-38), emerges 6 to 24 months after irradiation and
369 continues to progress over a period of years (39). The pathological mechanisms of radiation-induced

370 pulmonary fibrosis are complex and involve numerous cell types. A large number of studies have
371 presented evidence of the involvement of several mediators in the pathogenesis of this disease (36, 40).
372 A complex genetic background with a number of variations throughout the entire genome underlies
373 variability in response to treatments. The success of individual radiotherapy depends on complex
374 profiles, however, recent advances genotyping technology coupled with bioinformatics could offer new
375 opportunities to identify genetic biomarkers of normal tissue radiosensitivity (41). New approaches have
376 been developed with the advent of Next Generation Sequencing, which allows the sequencing in parallel
377 of millions of DNA fragments obtained either from the entire genome, the so-called genome-wide
378 association analysis (GWAS), or only from the coding fraction of the genome, the Exome, i.e Whole
379 Exome Sequencing (WES).
380 Currently, neither WES analysis nor other OMICs technologies (i.e. transcriptome, proteome), have been
381 used to assess toxicities associated to radiotherapy in lung/pleural cancer. It is important to note that
382 population-based studies can be difficult to translate into clinical care, especially in the case of a
383 multifactorial disease such as MPM in which surgical and radiotherapy procedures are not standardized
384 (12) and little is known about the genetic bases leading to augmented susceptibility to this disease (42-
385 44).
386 In our study we performed whole exome sequencing coupled with bioinformatic analysis to study
387 pulmonary toxicities in a group of MPM affected individuals who received the same treatment: non
388 radical surgery and neoadjuvant chemotherapy, followed by high dose radiation therapy. Patients were
389 divided into two groups according to the severity of the side effects developed after radical
390 radiotherapy on the entire hemithorax: none or tolerable pulmonary toxicity (NoSTox: Grade 0 to 2) and
391 more severe toxicities (STox: Grade 3).

392 By employing this approach, we aimed not only at identifying genetic variants associated with the
393 outcomes after radiotherapy, but also at unraveling the biological pathways potentially involved in the
394 resulting observed phenotypes.

395 Despite the genetic differences found among NoSTox and STox, principal component analysis (PCA)
396 showed a homogeneous distribution of variants in both groups. Thus, no global genetic specific
397 signature was identified in NoSTox and STox.

398 Here we describe the different pathways we found to be associated with the radiotherapy outcomes.

399 **NoSTox group**

400 Variant Enrichment Analysis revealed that the pathways characterizing the NoSTox group are mostly
401 involved with RNA processing, cell signaling, cell cycle and lipid synthesis; interestingly, also the TNFR1-
402 induced NFkappaB signaling pathway (R-HSA-5357956), appears to be involved.

403 Pulmonary irradiation can produce large amounts of reactive oxygen and nitrogen species that cause
404 oxidative damage to DNA, lipids and proteins (45). The resulting tissue injury triggers an inflammatory
405 reaction, which in turn leads to the secretion of proinflammatory factors that maintain and amplify the
406 R-HSA-5357956): a frameshift insertion in the UBC gene (chr12: 124911720) and a nonframeshift
407 deletion in the ovarian tumor protease 7B (OTUD7B) gene (chr1:149944731). The proteins encoded by
408 these genes are a ubiquitinase (Ubiquitin C) and a deubiquitinase (Deubiquitinase 7B), respectively.
409 Although overall changes in ubiquitin cellular levels in response to ionizing radiation are still little known
410 (opposite poorly understood), Tang and colleagues showed that down-regulation of ubiquitin C reduces
411 radiation-induced expression of NF- κ B and inhibits its translocation into the nucleus (55), suggesting
412 that upon irradiation, ubiquitin silencing may suppress NF- κ B-induced cellular response to radiation.

413 The deubiquitinase OTUD7B is a negative regulator of the non-canonical NF- κ B pathway (56), the
414 activation of which is characteristically slow and persistent. Typical inducers are ligands of a
415 subset of tumour necrosis factor receptor (TNFR) superfamily members. Largely on the basis of

416 animal models studies, non-canonical NF- κ B signalling has been implicated in the pathogenesis
417 of several inflammatory diseases (57). Overexpression of another member of the ovarian tumor
418 proteases (OTUs), OTUD4, which also negatively regulates inflammatory signalling (58), has been
419 shown to increase radiosensitivity in non small cell lung carcinoma (59).

420 In addition, also the AIM2 inflammasome pathway (R-HSA-844615) has been shown to have a potential
421 impact on NoSTox patients' phenotypes, with a frameshift deletion in the AIM2 gene (ENTREZID: 9447;
422 exon 5: c.712delA or p.T238Hfs*14; without rs ID; located on chr1 at 159062697 position) in patient
423 #16; also, a mutation on exon2 (c.C278G) of PYCARD (ENTREZ ID: 29108; mutation without rs ID located
424 on chr 16 at 31202200 position) was detected in patient #84.

425 – AIM2 is an innate immune sensor that mediates assembly and activation of the inflammasome in
426 response to double-stranded DNA breaks (60-61). In a rat model of radiation pneumonitis (RP),
427 radiotherapy increased the mRNA expression level of AIM2, which further triggered the release
428 of IL-1 β and induced RP (53), suggesting that the activation of the AIM2 inflammasome by
429 radiotherapy may contribute to the pathogenesis of RP. Interestingly, AIM2 inflammasome
430 deficiency has been shown to protect mice from radiation-induced small intestine syndrome as
431 well as hematopoietic failure (62).

432 The Insulin receptor signalling cascade (R-HSA-74751) is the pathway involved in the greatest number of
433 patients (n=4) who did not develop severe pulmonary toxicities (NSTox group).

434 – There is a high degree of structural homology between the insulin receptor (IR) and the insulin-
435 like growth factor 1 (IGF-1) receptor (IGF-1R), thus these receptors signal via many common
436 mediators (63). Ionizing radiation activates several tyrosine kinase receptors involved in DNA
437 damage response, including IGF-1R (64),
438 possibly promoting a pro-fibrotic state. Furthermore, dysregulation of the insulin-like growth
439 factor (IGF) axis has been implicated in the pathogenesis of fibrosis in lung diseases (65-68).

440 Insuline-like growth factor 2 (IGF-2) promotes fibrosis through IGF1R, IR, and IGF1R/IR,
441 differentiates fibroblasts into myofibroblasts, decreases protease production and extracellular
442 matrix degradation, and stimulates expression of two Transforming Growth Factor β (TGF β)
443 isoforms, suggesting that IGF-2 exerts pro-fibrotic effects via multiple mechanisms (69).

444 Recent evidence suggests that cholesterol-sphingolipid rafts might play a role in insulin signaling via IR
445 (70). The potential contribution of lipids to insulin signaling is intriguing since the Plasmalogen
446 biosynthesis (R-HSA-75896) pathway has also been shown to have a potential impact on NoSTox
447 patients' phenotypes, carried by patients #50, #66 and #88 who did not develop severe pulmonary
448 toxicities.

449 Plasmalogens represent a class of phospholipids ubiquitously found in considerable amounts as
450 constituents of mammalian cell membranes and significantly enriched in lipid rafts (71, 72), functional
451 microdomains in cell membranes, which may affect signal transduction. Therefore, disrupting
452 plasmalogen biosynthesis could affect trans-membrane signalling of insulin and IGFs, thus making a
453 functional bridge between these two genes and the pathogenetic mechanisms of radiation toxicity.

454 Furthermore, Laiakis and colleagues (73) investigated the effects of ionizing radiation on the blood level
455 of metabolites and lipids, showing that acute exposure to γ radiation in a mouse model induced specific
456 mobilization of plasmalogens. Though the pathological role of plasmalogens and the biochemical
457 pathways underlying their upregulation after radiation exposure remain to be elucidated, Braverman
458 and Moser suggested a specific serum lipidomic biosignature as an indicator of radiation exposure (74).

459 Coupling genetic signature to monitoring such a molecular response to radiation exposure, might have
460 implications not only for radiation pathology but also for countermeasures and personalized medicine.

461 Taken together these data from the NoSTox group, suggest that functional impairment in the control of
462 the radiation induced expression of NF- κ B, mainly in/of the ubiquitination/deubiquitination
463 mechanisms, together with an impairment of the AIM2 inflammasome pathway and a dysregulation of

464 the insulin-like growth factor (IGF) axis/plasminogen biosynthesis, could modulate radiation-induced lung
465 inflammation preventing development of acute radiation pneumonitis and chronic radiation-induced
466 pulmonary fibrosis.

467

468 **STox group**

469 In the attempt to unravel the adverse events in the STox group, Variant Enrichment Analysis and HPO
470 were performed in order to establish a correlation between genetic background and clinical phenotype
471 of the patients.

472 Variant Enrichment Analysis

473 As far as the group of individuals who developed severe pulmonary toxicities is concerned (STox), a
474 pathway, with potential impact on these patients' phenotypes was found: the Dissolution of the Fibrin
475 Clot pathway (RHSA-75205). Only one mutation related to this this pathway was identified: a stopgain
476 mutation in the SERPINF2 gene (rs374446894) in patient #2, who developed a thromboembolic event.

477 – Irradiation activates procoagulant activity and reduces fibrinolytic activity (75) in animal models
478 of thoracic irradiation, suggesting the involvement of coagulation and fibrinolysis in radiation-
479 induced pulmonary inflammation, with activation of the coagulation system promoting
480 pulmonary fibrin deposition and contributing to the progression of pulmonary injury (76).
481 Reduced fibrinolytic activity in the irradiated lung could be ascribed to both decreased
482 plasminogen activator activity (75, 77) and increased activity of its inhibitor. The SERPINF2 gene
483 (rs374446894) encodes a member of the serpin family of serine protease inhibitors, the alpha 2
484 antiplasmin (α 2-AP), which, together with the plasminogen activator inhibitor-1 (PAI-1), is the
485 principal direct inhibitor of fibrinolytic proteases such as plasmin, that degrades fibrin and
486 various other proteins. Thus, α 2-AP and PAI-1 play a major role in regulating the blood clotting
487 pathway. PAI-1 also blocks the degradation of the extracellular matrix (78) and has been shown

to be implicated in the progression of inflammatory and fibrotic lung diseases. Several studies have demonstrated the involvement of PAI-1 in radiation-induced tissue injury and fibrosis (79, 80). The overexpression of PAI-1 has been reported to lead to the development of radiation-induced nephrosclerosis (81, 82) and enteritis (83).

A recent study demonstrated that a truncated PAI-1 protein (rPAI-123) protects against radiation-induced lung injury in a murine model (84). Furthermore, PAI-1:rs7242 in the 3'-untranslated region of PAI-1, has been shown to be a predictor of grade ≥ 3 radiation pneumonitis before radiotherapy (85). A recent study on radiation-induced lung fibrosis in PAI-1 knockout mice has suggested an important role of PAI-1 in radiation-induced pulmonary fibrosis (86).

One large study, involving 254 women treated with breast conserving therapy, 31 of whom developed fibrosis after RT, identified 87 differentially expressed genes. Among these, the up-regulation of SERPINE1, coding for PAI-1, was found (87), confirming the increased protein levels observed in fibrotic conditions (78).

PAI-1 seems to play an important role in radiation-induced tissue injury (Shioya et al, 2018), being involved in both the RP and lung fibrosis process. Likewise, Alpha 2 antiplasmin ($\alpha 2$ -AP), encoded by the SERPINF2 gene, which shares most of its functions with PAI-1, could also be involved in the radiation-induced pulmonary injury observed in patient #2, who developed both a pulmonary thromboembolic event and pneumonitis.

Interestingly, a high impact non-synonymous mutation in the ADAR gene, involved in the formation of editosomes by ADAR proteins, was carried by patient #94, who progressed to high grade fibrosis.

- The post-transcriptional modification of RNA is a key process controlling the output of the genome, and the deamination of adenosines (A) to inosines (I) is the prominent RNA editing event in humans, catalyzed by the Adenosine Deaminase Acting on RNA (ADAR) family of proteins (88). The effects of radiation on RNA editing are poorly understood, Liu and colleagues

reported that upon/after α -particles radiation, RNA editing sites change greatly and their total amount decreases after radiation (89). It has been determined that the expression of ADAR is tissue-specific, the lung being the second most highly expressing site, in terms of tissue expression (90, 91). The RNA editome (R-HSA-77042) analysis of idiopathic pulmonary fibrosis (IPF) and normal lungs revealed increased editing frequency in IPF compared to normal lungs, suggesting a role for dysregulated editing in IPF pathogenesis (92). It has been found that the expression of ADAR1 and 2 is downregulated in fibroblasts from patients with IPF (93). The changes in expression levels of ADAR1 and ADAR2 may represent an important controlling mechanism in IPF, regulating the processing of key miRNAs such as miRNA-21. Overexpression of miRNA-21 in lung tissue and pulmonary fibroblasts from patients with IPF may be due to defective editing by ADAR (93). This miRNA targets anti-fibrotic proteins such as SMAD family member 7 (small mother against decapentaplegic), TGF β receptor 2 (TGF β R2), TIMP Metalloproteinase Inhibitor 3 (TIMP3), Vascular endothelial growth factor A (VEGF-A) (94, 95). Crosstalk among impact pathways which control RNA editing (R-HSA-75064 and R-HSA-77042) could contribute to the induction of the severe lung fibrosis observed in patient #94.

Overall, these results suggest that, by affecting both the fibrinolytic activity and RNA editing pathways, irradiation could be responsible of the severe toxicity events reported by some patients of the STox group, who present specific mutations in genes involved in these pathways.

HPO analysis

Furthermore, in an attempt to unravel the adverse events in the STox group, HPO was performed to establish a correlation between genetic background and clinical phenotype of the patients. This approach aimed at assessing, for each patient characterized by different outcomes, the genes and number of variants associated with the clinical phenotypes (i.e Dyspnea, Fibrosis, Pneumonitis and Thromboembolic events). HPO, however, did not provide conclusive evidence, since most of the variants

536 observed were characterized by low CADD score and thus didn't have great impact on the genes. In
537 addition, variants in the myopalladin gene (MYPN) were found to be associated with thromboembolic
538 events in three patients (#2, #17 and #70). However, so far this gene has been associated with dilated
539 cardiomyopathy (27), while no associations have been reported with lung thromboembolism, so the
540 significance of this association remains obscure.

541 Finally, PCA was performed with the objective of clusterizing both NoSTox and STox patients according
542 to their genetic characteristics. Even if genetic differences were observed between the NoSTox and STox
543 groups, PCA showed a very homogeneous distribution, with no clusters related to either the NoSTox or
544 STox clinical groups. Therefore, a specific signature typical of the two groups could not be detected.
545 Patient #53 (STox) was the only one to be very distant from the NoSTox and STox clusters, without
546 showing any specific phenotypic characteristics, so we can hypothesize that his genetic background, not
547 related to toxicity, was simply quite distant from the other individuals analyzed, without repercussions
548 on the studied phenotypes.

549 Recently, GWAS results have identified SNPs correlated with side-effect after radiotherapy in patients
550 affected by different cancers, such as breast, prostate and lung. In our whole exome sequencing (WES)
551 study we identified crucial pathways driving the lung response to ionizing radiations in patients affected
552 by mesothelioma. Even if we cannot present a unifying view of the mechanisms that determine
553 radiation toxicity of the lung, in this paper we report evidence for the key role of some specific
554 pathways, the relevance of which is also supported by a number of published studies. They can
555 therefore represent important starting points for more focused future investigations. Nevertheless, we
556 are aware that our study suffers from the limitation of the small number of patients analysed, thus the
557 results need to be validated on larger sample of patients. Moreover, RNA from patient tissues (lungs,
558 pleura etc.) was not available, so the pathways we indentified in our study could not been further
559 validated by RNASeq analysis.

560 CONCLUSION AND FUTURE PERSPECTIVES

561 In conclusion, our preliminary results could pave the way for the definition of a genomic signature, i.e. a
562 panel of predictive genomic variants, capable of supporting the management of MPM patients. By
563 allowing for early identification of patients at high risk for treatment-dependent pulmonary toxicity, this
564 predictive tool could play a major role in the design of new therapeutic combinations, including
565 immunotherapy and RT. Indeed, first line nivolumab plus ipilimumab treatment has been recently
566 proposed in unresectable MPM, to improve OS compared to chemotherapy The potential synergy of RT
567 and immunotherapy, already observed in other cancers (96), could further improve the clinical response
568 in MPM, but also increase the risk of side effects, shared by both treatments, such as pneumonitis (97).
569 In this context, the pre-treatment genomic characterization we propose in this paper could help to
570 prevent the development of severe side effects by contributing to the definition of a personalized
571 treatment.

572

573 **Abbreviations**

574 MPM: Malignant pleural mesothelioma; P/D: Pleurectomy/Decortication; OS: Overall Survival; SNPs:
575 single nucleotide polymorphisms; GWAS: Genome-Wide Association analysis; PET: Positron Emission
576 Tomography; CT: Computed Tomography; RHR: Radical Hemithoracic Radiotherapy; PCA: Principal
577 Component Analysis; HPO: Human Phenotype Ontology; NoSTox: No severeTox; STox: SevereTox; GTT:
578 gamma-glutamyltransferase; ExonVars: Exonic Variants; FunctVar: Functional Variants; ImpactVar:
579 Impact Variants; MYPN: Myopalladin gene; TNF α : Tumor Necrosis Factor α ; TNFR: Tumour Necrosis
580 Factor Receptor; OTUs: Ovarian Tumor Proteases; OTUD7B: Ovarian Tumor Protease7B; AIM2: Absent in
581 Melanoma 2; SERPINF2: Serpin Family F Member 2; ADAR: Adenosine Deaminase RNA Specific; UBC:
582 Ubiquitin C; CADD: Combined Annotation Dependent Depletion; IR: Insulin Receptor; IGF-1: Insulin-like
583 Growth Factor 1; IGF-2: insuline-like growth factor 2; TGF β : Transforming Growth Factor beta; α 2-AP:

584 alpha 2 antiplasmin; PAI-1: Plasminogen Activator Inhibitor-1; IPF: Idiopathic Pulmonary Fibrosis; RP:
585 Radiation Pneumonitis.

586

587 **DECLARATIONS**

588 ***Ethics approval***

589 *The study has been declared to and accepted by the local Ethical Committee (Comitato Etico*
590 *Indipendente del CRO di Aviano, CRO-2013-38) and written informed consent was obtained from all the*
591 *patients. The CRO-biobanking service managed and stored all biological samples before use for the*
592 *present project (authorization for analyses obtained through protocol number 6825/D).*

593

594 ***Consent for publication***

595 *Not applicable.*

596

597 ***Availability of supporting data***

598 *The datasets supporting the conclusions of this article are included within the article and its additional*
599 *file see Supplementary section*

600

601 ***Competing interests***

602 *The authors declare that they have no competing interests.*

603

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608

609 ***Authors' contributions***

610 *SC and VB conceived the original idea and drafted the manuscript, AR acquired the data and contributed*
611 *to the interpretation of the results, RRM and LB performed genetic analysis and interpretation of the*
612 *data, EM and GZ contributed to the final version of the manuscript, provided critical feedback and helped*
613 *shape the research, AS, MT, EM and PZ helped supervise the project. All authors read and approved the*
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615

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956 **Supplementary**

957 **Additional datasets supporting the conclusions of this article (Supplementary Table 1.-5) are included**
958 **within these additional files: Supp_table_1.xls and Suppl_tables_2_3_4_5.xlsx**

959

960 **Supplementary Table 1.**

961 Legend: Excel file (**Supp_table_1.xls**) with 4 datasheet.

962 Datasheet 1-Exclusive FunctVar STox: List of 43 FunctVar exclusive for STox group.

963 Datasheet 2-Exclusive ImpactVar STox: List of 338 ImpactVar exclusive for STox group.

964 Datasheet 3 – Genes with FunctVar STox: List of 476 FunctVar from 33 genes exclusive from STox group.

965 Datasheet 4 - Genes with ImpactVar STox: List of 937 ImpactVar from 225 genes exclusive from STox
966 group.

967 Variant ID (vi) without rs were described by vi crhomosome and position (vi17.69277741; i.e. variant in
968 chromosome 17 located on 69277741). Damage prediction were calculated by the mutation score of :

969 SIFT, Polyphen2, LRT, Mutationtaster, Mutationassessor, FATHMM, Provean, Metasvm, Metalr and

970 M_cap databases. Allele frequency (AF) were accessible by GnomAD Exome 3.0.

971

972 **Supplementary Tables 2, 3, 4 and 5.**

973 Legend: Excel file (**Suppl_tables_2_3_4_5.xlsx**) with 4 datasheet

974 Datasheet 1- Supplementary Table 2. List of the variants present in the functional pathways found in the
975 non-toxicological effect group.

976 Datasheet 2- Supplementary Table 3. List of the variants present in the functional pathways found in the
977 toxicological effect group.

978 Datasheet 3- Supplementary Table 4. List of the variants present in the impact pathways found in the
979 non-toxicological effect group.

980 Datasheet 4 - Supplementary Table 5. List of the variants present in the impact pathways found in the
 981 toxicological effect group.

982

Supplementary Table 6. HPO analysis of STox pulmonar grade 3 phenotypes: Dyspnea (D), Fibrosis (F), Pneumonitis (P) and Thromboembolic Event (TE).									
Patient ID#	<6 months				>6 month				N° ImpactVar related to HPO database phenotypes
	D	F	P	TE	D	F	P	TE	
2			X	X	X				4 Impactvar at the myopalladin gene (MYPN*) were associated to Thromboembolic event
12		X							2 ImpactVar, along 2 genes**, were associated to the pneumonia
17								X	25 ImpactVar, along 12 genes related to fibrotic phenotypes
20							X		4 Impactvar at the myopalladin gene (MYPN*) were associated to Thromboembolic event
53							X		2 ImpactVar, along 2 genes**, were associated to the pneumonia
58	X				X		X		No association
61							X		2 ImpactVar, along 2 genes**, were associated to the pneumonia
70			X	X	X				No association
94						X			4 Impactvar at the myopalladin gene (MYPN*) were associated to Thromboembolic event
									2 ImpactVar, along 2 genes**, were associated to the pneumonia
						X			25 ImpactVar, along 12 genes related to fibrotic phenotypes

983 *No association with lung thromboembolism
 984 **CADD_Score predicting low impact on the protein.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supptable1.xls](#)
- [Suppltables2345.xlsx](#)