

1 **Title:**

2 Proposal for a combined histo-molecular algorithm to distinguish multiple primary  
3 adenocarcinomas from intrapulmonary metastasis in patients with multiple lung tumors.

4

5 **Short title:** Multiple lung adenocarcinomas classification

6

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9

## 1 **Abstract**

2  
3 **Introduction:** Multiple nodules in lung are diagnosed with an increasing frequency due to  
4 high quality CT-scan imaging. In patients suffering from lung cancer, this situation represents  
5 up to 10% of operated patients. For clinical management, it is important to classify the disease  
6 as intra-pulmonary metastasis or multiple primary lung carcinomas to define TNM  
7 classification and optimize therapeutic options. In the present study, we evaluated the  
8 respective and combined input of histological and molecular classification in order to propose  
9 a classification algorithm for multiple nodules.

10 **Methods:** we studied consecutive patients undergoing surgery with curative intent for lung  
11 adenocarcinoma (n=120) and harboring two tumors (n=240). Histological diagnosis according  
12 to the WHO 2015 classification and molecular profiling using a 22 hotspot genes targeted  
13 NGS allowed classifying samples as multiple primary lung adenocarcinomas (MPLA) or as  
14 intra-pulmonary metastasis (IPM).

15 **Results:** NGS identified molecular mutations in 91% (n=109/120) of tumor pairs. Genomic  
16 and histologic classification showed a fair agreement using kappa test ( $K = 0.43$ ). Discordant  
17 cases (n=30/109, 27%) were reclassified using a combined histo-molecular algorithm. *EGFR*  
18 mutations ( $p=0.03$ ) as well as node involvement ( $p=0.03$ ) were significantly associated to  
19 IPM, whereas *KRAS* mutations ( $p=0.00005$ ) were significantly associated to MPLA. *EGFR*  
20 mutations ( $p=0.02$ ) and node involvement ( $p=0.004$ ) were the only independent prognostic  
21 factors.

22 **Conclusion:** We showed that combined histo-molecular algorithm represents a relevant tool  
23 to classify multifocal lung cancers, which could guide adjuvant treatment decisions. Survival  
24 analysis underlines the good prognosis of *EGFR* mutated adenocarcinoma in patients with  
25 intra-pulmonary metastases.

26  
27 **Keywords:** multiple lung cancer, intrapulmonary metastasis, histological and molecular  
28 classification, NGS.

## 1 **Introduction**

2

3 The improvement of lung cancer detection by CT-scan has increased the frequency of  
4 multiple pulmonary nodules situation, representing up to 10% of all lung cancers.<sup>1-3</sup> Multiple  
5 nodules may be synchronous when diagnosed at the same time, or metachronous since  
6 patients with lung cancer have a 3.5 times higher risk of developing a second lung cancer.<sup>4</sup>  
7 Patients with independent primary tumors are expected to have a better prognosis than  
8 patients with intra-pulmonary metastasis (IPM).<sup>5-7</sup> Martini and Melamed proposed the first  
9 classification of multiple lung nodules to distinguish multi-primary carcinoma from IPM,<sup>8</sup>  
10 based on clinical and pathological data. However, this empirical classification does not take  
11 into account the latest WHO 2015 classification and does not include molecular analysis  
12 which is now routinely performed for lung adenocarcinoma.<sup>9-11</sup> Since the previous TNM  
13 classification,<sup>12-14</sup> lung cancer are classified T3 if more than one nodule is found in the same  
14 lobe, T4 when found in a different but homolateral lobe and M1a when found in the  
15 contralateral side. However, it is based on the assumption that multiple nodules are IPM.  
16 Therefore, classifying multi-nodular lung cancer disease is important because the therapeutic  
17 management may change from no adjuvant treatment to multimodal therapy combining  
18 radiotherapy-chemotherapy-immunotherapy. Histological classification and clinical data are  
19 not themselves fully satisfactory to identify IPM, and molecular profiling has been assessed in  
20 this setting. Loss of heterozygosity,<sup>15-17</sup> genomic profiling using CGH<sup>2</sup> and genomic  
21 rearrangement profiles<sup>18</sup> were shown to be efficient in classifying tumors but could be hard to  
22 implement in routine practice. Recently, a concordance of 70% was found between  
23 morphologic and molecular classification in lung adenocarcinoma using 4 drivers  
24 oncogenes.<sup>19</sup> Oncogenic driver mutations appear early in carcinogenesis with a limited intra-  
25 tumor heterogeneity,<sup>20-23</sup> but these frequent alterations might also be shared between  
26 independent tumors. Thus, the implementation of next-generation sequencing (NGS)  
27 enhances the accuracy of molecular testing to distinguish multiple primary lung  
28 adenocarcinoma (MPLA) from IPM.<sup>24-26</sup> Here, we studied 120 patients harboring two  
29 synchronous or metachronous lung adenocarcinomas and report a proposal for histo-  
30 molecular algorithm to classify patients as IPM or MPLA.

31

## 1 **Materials and methods**

2

### 3 *Patients and tumor selection*

4 Between January 2010 and December 2012, 1173 lung carcinoma patients were operated on  
5 at Cochin hospital, Paris, France, 136 (11.6%) of these patients had more than one lung  
6 carcinoma, including 120 patients who presented two adenocarcinomas with sufficient  
7 material for histological and molecular analysis. Patients had synchronous tumors or  
8 metachronous, defined by a second tumor occurring at least 6 months after the first one.  
9 Patients who received neo-adjuvant therapies and those with extra-thoracic metastasis were  
10 excluded. Overall survival (OS) was retrospectively obtained. This study was approved by the  
11 local ethic committee (CPP Ile de France II, n°2008-133, n°2012 06-12 and 2018 MS1).

12

### 13 *Histological assessment*

14 All tumor slides were reviewed (range 1 to 9 according to tumor size) by two experienced  
15 lung cancer pathologists (ALM, DD), in order to re-classify adenocarcinoma according to the  
16 latest WHO classification (2015),<sup>27</sup> depending on predominant architectural pattern. For each  
17 tumor, the percentage of each histological component (architectural pattern) was semi-  
18 quantitatively recorded with 5% increments for each slide. The predominant pattern was  
19 defined as the pattern with the largest percentage and was grouped into low, intermediate and  
20 high prognostic grades, as previously described.<sup>9,28</sup> Histological criteria for distinguishing  
21 IPM from MPLA were adapted from Detterbeck<sup>30</sup> and Nicholson,<sup>31</sup> and consisted of: (a)  
22 architectural pattern; (b) cytologic features (cell size, cuboid or cylindrical cells, nuclear  
23 pleomorphism, nuclear inclusion, nucleolar size, mitotic rate, estimated amount of cytoplasm,  
24 quantity of cells with mucin); (c) tumor stroma (cellular or not, estimated number and type of  
25 immune cells); and (d) TTF1 expression (SP24 clone, Novocastra). We proposed an algorithm  
26 detailed in Figure 1A to classify, using histological criteria, tumor pairs as multiple primitive  
27 lung adenocarcinomas (MPLA) or as intrapulmonary metastatic (IPM). *In situ*  
28 adenocarcinoma (AIS) and minimally invasive adenocarcinoma (MIA) were always  
29 considered as MPLA, as a recent publication suggests that they are genetically independent.<sup>14</sup>  
30 Localization (same, different lobe or contralateral), emboli, pleural invasion and lymph node  
31 status were not taken into account to classify tumors histologically.

32

1 *Martini and Melamed classification*

2 All multiple lung adenocarcinoma patients were classified as MPLA and as IPM with the  
3 reference Martini and Melamed classification published in 1975.<sup>8</sup> This classification is based  
4 on histological type, ie adenocarcinoma or squamous cell carcinoma but the histological  
5 subtyping of adenocarcinoma, as described in the WHO's classification was not taken into  
6 account since it has been defined recently. In Martini and Melamed classification,  
7 synchronous tumors exhibiting similar histological type with no lymphatic or systemic  
8 metastases were classified as MPLA when they were located in different segments.  
9 Metachronous tumors were classified as MPLA when time lapse between tumors was over 2  
10 years; as IPM when time lapse between tumors was less than 2 years with both tumors in the  
11 same lobe as IPM when time lapse between tumors was less than 2 years and tumors were in  
12 different lobes with lymph node involvement or systemic metastasis.

13

14 *Molecular analysis*

15 For each tumor, DNA was extracted from the FFPE block containing the highest percentage  
16 of tumor cells (at least 30%). DNA extraction and NGS analysis was performed as previously  
17 described,<sup>29</sup> using the (Ion AmpliSeq™ Colon-Lung Cancer Research Panel v2 (Life  
18 Technologies™, France), covering more than 500 hotspot mutations within 22 genes (*KRAS*,  
19 *EGFR*, *BRAF*, *PIK3CA*, *AKT1*, *ERBB2*, *PTEN*, *NRAS*, *STK11*, *MAP2K1*, *ALK*, *DDR2*,  
20 *CTNNB1*, *MET*, *TP53*, *SMAD4*, *FBXW7*, *FGFR3*, *NOTCH1*, *ERBB4*, *FGFR1* and *FGFR2*),  
21 using Ampliseq technology (Ion ampliseq library kit V2, Ion library equalizer kit, Life  
22 Technologies™), then clonal amplification on the Ion Chef™ System (ION P1 HI-Q CHEF,  
23 ION PI CHIP KITV3) and sequencing (Ion Proton system). Torrent Suite 5.0.4 was used to  
24 analyze data. VCF files from the variant caller were annotated using the Safir2report tool.  
25 Quality criteria used as end-points were a detection threshold of 2% and a minimum coverage  
26 depth of 300X. For each patient, concordance of polymorphisms between all tumors was  
27 verified. A minimum coverage depth of 300X was required to validate the variants. NGS  
28 coverage depth data were used to identify gene amplifications using an algorithm locally  
29 developed based on the identification of outliers from the expected coverage mean + 3SD.<sup>29</sup>  
30 As, gene amplifications were not taken into account to classify tumors because they might be  
31 missed by targeted NGS when heterogeneous or might be acquired during tumor  
32 progression.<sup>23</sup> Thus, the link between both adenocarcinomas was determined according to the  
33 concordance of somatic molecular mutations as follows: -1- multiple primary lung

1 adenocarcinoma (MPLA) when tumors had no mutation in common or when tumors had  
2 different driver mutations in *EGFR*, *KRAS*, *BRAF*, *ERBB2*, *NRAS* or *MET* genes ; -2-  
3 metastatic tumor (IPM) when all alterations were common between tumors ; -3- IPM with  
4 additional alteration (IPM w AA) when at least one mutation was shared between tumors with  
5 one or more additional alterations in one of the samples, including amplification; -4-  
6 undetermined when both tumors were wild type. Silent (n=13), amplification (N=24) and  
7 intronic alterations (n=9) were not considered as classifiers. Tumors were classified  
8 independently from the histological analysis and clinical data.

### 9 10 *Proposed algorithm for classifying multiple lung tumors*

11 This algorithm, based on integrated clinical and histo-molecular criteria (Figure 1B) defines  
12 the final classification. Firstly, tumors were classified as MPLA when one nodule presented as  
13 either an *in situ* adenocarcinoma or a minimally invasive adenocarcinoma. We chose a  
14 threshold of 5 years to consider metachronous nodules as MPLA. Secondly, tumor pairs were  
15 classified as MPLA or as IPM according to molecular results, except for tumor pairs that  
16 harbored the same frequent mutation in *KRAS* (p.G12X) or *EGFR* (del19 or p.L858R) genes  
17 that were classified according to the histological classification, defined by the histological  
18 algorithm (Figure 1A). Wild type tumor pairs, without AIS or AIM in one tumor and/or  
19 metachronous >5 years, were classified undetermined. The final classification was compared  
20 with Martini's one.”

### 21 22 *Statistical analyses*

23 Clinical, pathological and molecular characteristics were collected using Research Electronic  
24 Data Capture (REDCap).<sup>30</sup> Variables were compared using a Student's t-test (continuous) or  
25 chi-squared test (categorical). Level of agreement between classifications was assessed by  
26 Cohen's kappa.<sup>31</sup> Kaplan-Meier plots, log-rank and Cox proportional hazards regression were  
27 used to describe the effect of variables on survival in univariate analysis. Multivariate analysis  
28 was performed using a Cox model for variables significantly associated with OS (p-  
29 value < 0.05) except for Martini's classification, which is dependent on nodal status. Analyses  
30 were performed with R software (<http://www.r-project.org/>) (R version 3.4.3) using the  
31 'survival' (version 2.41-3) and the 'survminer' (version 0.4.2) packages.<sup>32</sup>

# 1 **Results**

2

## 3 *Patient and histological characteristics*

4 A total of 120 patients, predominantly men (71; 59%) and smokers (105; 87%) were included  
5 in this study (Table 1), 86 patients (72%) had synchronous tumors and 34 metachronous  
6 tumors. Forty-two patients (35%) had a lymph node-positive disease (19 N1 and 23 N2).  
7 Among synchronous patients, 43 (50%) had both tumors in the same lobe, 35 (41%) in  
8 another ipsilateral lobe and 30 (35%) had a lymph node-positive disease (18 N2 and 12 N1).  
9 Among metachronous patients, 17 (50%) were on the same side among which 7 in the same  
10 lobe; and 6 (18%) had lymph node-positive disease in the first tumor. The three most  
11 frequently predominant architectural pattern were tubular (N=91, 38%), papillary (N=59,  
12 25%), and solid (N=48, 20%) (Table 1). Tumor emboli were found in 124 tumors (52%) and  
13 87 had pleural infiltration (36%).

14

## 15 *Tumor molecular characteristics*

16 A total of 340 genetic mutations were identified in these 240 tumors (Supplementary-Figure  
17 1). At least two mutations were found in 90 tumors (38%) and no mutation was identified in  
18 39 tumors (16%). Frequently mutated genes were *TP53* (n=91), *KRAS* (n=90; p.G12C n=38),  
19 *EGFR* (n=31; exon 19 deletion n=15; p.L858R n=10; exon 20 insertion n=2 and rare  
20 mutations n=4), *STK11* (n=17) and *BRAF* (n=16). *KRAS* mutations were not associated with  
21 *EGFR*, *ERBB2* or *MET* activating alterations. Five tumors harbored co-driver mutations  
22 involving *BRAF* with another potential driver. Gene amplifications (e.g. *EGFR*, *KRAS*,  
23 *FGFR1* and *ERBB2*) were identified in tumor samples from 20 patients but were only present  
24 in paired tumors for 4 patients.

25

## 26 *Classification of patients into intra-pulmonary metastatic (IPM) or multi-primitive lung 27 adenocarcinoma (MPLA)*

28 Histological classification identified 56 IPM and 64 MPLA patients, while 39 IPM, 70 MPLA  
29 and 11 undetermined cases (both tumors being wild type) were defined by molecular analysis  
30 (Supplementary-Figure 2). Molecular and histological classifications were concordant for  
31 79/109 (72%) patients (29 IPM and 50 MPLA) and discordant for 30 cases (10 MPLA-  
32 histology/IPM-molecular and 20 IPM-histology/MPLA-molecular) with a final kappa score of  
33 0.43 (Figure 2). Among the 10 MPLA-histology/IPM-molecular discordant patients, IPM



1 molecular classification relied on the presence of a shared hotspot mutation (5 *KRAS* p.G12X)  
2 for 6 patients, and two shared alterations for 3 patients. Among the 20 IPM-histology/MPLA-  
3 molecular discordant patients all, except one, had a frequent predominant architectural pattern  
4 either tubular, papillary or solid. The combined histo-molecular classification based on the  
5 algorithm proposed in Figure 1 allowed the classification of 111/120 patients with 33 IPM  
6 and 78 MPLA. IPM and MPLA patients differed significantly for: smoking history ( $p=0.007$ ),  
7 time lapse between tumors ( $p=0.03$ ), tumor location ( $p=0.0001$ ), node involvement ( $p=0.03$ )  
8 and mutational status for *EGFR* ( $p=0.03$ ) and *KRAS* ( $p=0.00005$ ) (Table 3, Supplementary-  
9 Figure 3).

10 Using Martini and Melamed classification, we identified 57 IPM and 63 MPLA. We  
11 compared our histo-molecular classification to that of Martini and Melamed and found  
12 discordance for 37 patients (Kappa score 0.31). Ten patients were MPLA-Martini/IPM-histo-  
13 molecular due to time to relapse  $>2$  years ( $N=3$ ), location in different segment ( $N=3$ ) or lobes  
14 ( $N=4$ ) and N0; 27 patients were IPM-Martini/MPLA-histo-molecular due to N+ disease  
15 ( $N=15$ ) and to nodules located in the same lobe/segment ( $N=12$ ).

16

### 17 *Follow-up and survival*

18 Survival information was available for 119 patients, 58 (49%) had died at the time of the  
19 study and among them one patient died within perioperative period (18 days after surgery)  
20 and was excluded from the survival analysis. Time to death was measured from the time of  
21 the first surgery in metachronous diseases. The median survival was 57.5 months [1.6-152.9].  
22 In univariate analysis, OS was significantly associated with nodal status ( $p=0.0001$ ), vascular  
23 tumor emboli ( $p=0.008$ ), *EGFR* mutations ( $p=0.03$ ) and Martini classification ( $p=0.01$ ) (Table  
24 4, Supplementary-Figure 4). Survival did not differ significantly between MPLA and IPM  
25 (molecular, histological and combined histo-molecular classification) (Table 4). Median  
26 survival times for MPLA and IPM patients, using our histo-molecular classification, were  
27 62.5 months [range: 1.6-152.9] and 56.5 months [range: 6.3-104.5], respectively. In  
28 multivariate analysis, *EGFR* status ( $p=0.02$ ) and nodal status ( $p=0.004$ ) remained independent  
29 prognostic factors.

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## 1 Discussion

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3 In the era of precision oncology, the context of multiple lung nodules has to be revisited.  
4 Here, we proposed an algorithm to classify multiple lung adenocarcinomas according to histo-  
5 molecular data to accurately define tumor stage and adapt therapeutics. Surgical samples from  
6 the patients of our study were analyzed at histological and molecular levels, and compared to  
7 Martini's classification. The studied population has similar histology and molecular profiles  
8 to that already described in adenocarcinoma regardless of the presence of multiple nodules.  
9 Interestingly, only 11 wild type paired tumors (9% of all patients) were found and therefore  
10 inconclusive for molecular classification using a NGS lung panel, whereas, up to 34 patients  
11 (28%) would have been classified "undetermined" if we had only looked at the 5 theranostic  
12 genes (*KRAS*, *EGFR*, *ERBB2*, *BRAF*, *MET*). In a previous study<sup>19</sup> analyzing only 4 drivers  
13 (*KRAS*, *EGFR*, *BRAF*, *ALK*), the frequency of inconclusive cases was 24%. The concordance  
14 between histological and molecular classification was moderate with a kappa score of 0.55, in  
15 agreement with our finding. This moderate agreement level is mainly due to frequent patterns  
16 in both classifications that are not robust classifiers: tubular, papillary and solid pattern in  
17 IPM-histology/MPLA-molecular and *KRAS* mutations p.G12X in MPLA-histology/IPM-  
18 molecular.

19 Molecular classification is based on the presence of a common driver as a marker of similar  
20 origin. This assumption may be discussed, particularly when a frequent driver alteration is  
21 used as a unique classifier. Indeed in our study, 6 patients had paired tumors with *KRAS*  
22 (p.G12C or p.G12V) or *EGFR* (p.L858R) mutations but we reclassified them as MPLA  
23 thanks to differences in the histological pattern. In smokers, tobacco injury may favor the  
24 development of multiple de novo primary cancers with independent *KRAS* mutations. This is  
25 in line with our observation of a significant association between *KRAS* mutations and  
26 MPLA.<sup>33</sup> In contrast, *EGFR* mutations were statistically related to IPM, suggesting that tumor  
27 cells may spread locally and/or form local clonal metastasis, which is already known.<sup>34</sup> The  
28 *TP53* gene mutation was an interesting discriminating factor due to its frequent involvement  
29 in lung cancer and to the absence of hotspot variants.<sup>16,17,35-38</sup> *TP53* mutation was the only  
30 classifier in 4 IPM patients and in 6 MPLA patients. However, *TP53* gene mutations were  
31 also reported to be implicated in clonal evolution within tumors.<sup>35</sup> In our analysis, among the  
32 14 metastatic tumors with additional molecular alterations, 4 were *TP53* mutated. Similarly,  
33 gene amplifications may be heterogeneous and acquired during tumor progression.<sup>23</sup> In our

1 study, we detected gene amplifications in 8 IPM patients among which 4 were present in  
2 tumor pairs. Concordant gene amplifications could be an additional argument to diagnose  
3 tumors as IPM. In case of gene amplification discordance, as seen in 4 IPM pairs, the  
4 amplification was probably acquired during progression. Finally, rare alterations represented  
5 an interesting and an additional way to differentiate IPM from MPLA, and we classified as  
6 IPM 4 tumors harboring *BRAF*, *NRAS*, *ERBB2* or *ERBB4* mutations.

7 Morphological assessment of architectural pattern is mostly contributive when the  
8 predominant patterns are rare, but tubular, papillary or solid patterns are so frequent that they  
9 can be observed even in different primary tumors, therefore it impairs some histological  
10 classification. Interestingly, different predominant pattern paired-tumors turned out to be IPM  
11 in 3 patients (Table 2), strengthening the fact that molecular assessment is mandatory to  
12 distinguish MPLA to IPM. We used standard of care methods for histological diagnosis and  
13 for molecular profiling in order to propose a “real life algorithm” for therapeutic guidance. In  
14 contrast to other publications which focused either on genetics or morphology,<sup>25,26</sup>  
15 comparison of histo-molecular classification to Martini’s showed poor agreement and may  
16 indicate the limits of Martini classification in several situations, as previously reported.<sup>39,40</sup>  
17 Indeed, clinical data such as nodal involvement has a strong impact on classifying IPM  
18 samples in Martini’s. Nevertheless, the lymph node status was not consistently helpful to  
19 classify MPLA. Here, we showed that 20 MPLA patients have node invasion and 13 IPM are  
20 N0. Moreover, like previous studies<sup>41</sup> we showed, that metachronous cancers could be IPM  
21 even when relapses occurred after 2 years, but never after 5 years.

22 Survival analysis showed that node and vascular involvements and *EGFR* mutations were  
23 statistically linked to OS. There are discrepancies concerning the prognostic value of *EGFR*  
24 mutation in patients with resected lung adenocarcinoma.<sup>42</sup> In our study, we analyzed a subset  
25 of operated patients having two lung nodules with *EGFR* mutations and this specific situation  
26 has not been clearly analyzed previously. However, one limitation of our study is the  
27 interpretation of the positive prognostic value of *EGFR* mutated tumors. Indeed, we found  
28 that among the 13 patients with *EGFR* mutated tumors with available clinical data, 6 received  
29 post-operative EGFR-TKI. This treatment may interfere with final *EGFR* mutations  
30 prognostic even if there is no OS significant difference for patients treated or not with EGFR-  
31 TKI (p=0.8). In contrast to Martini’s classification, which relies on nodal status, in our cohort  
32 neither histological nor molecular nor combined histo-molecular classifications were  
33 associated with OS. This deserves some discussion. Indeed, our results suggest that patients

1 with surgical resection for multifocal MPLA or IPM tumors have similar prognosis and that  
2 OS is mainly impacted by nodal status. This is now referred to as “oligo-metastatic” disease  
3 with patient reported to have similar survival as non-metastatic ones.<sup>43-50</sup> It may also reflect a  
4 bias in our study because only highly selected patients underwent surgical resection of  
5 multiple pulmonary nodules (PS 0-1). In agreement with others, we did not find any  
6 differences of OS between metachronous and synchronous tumors.<sup>7</sup> In a previous publication,  
7 Roepman *et al* showed a trend toward improved prognosis in MPLA patients but they did not  
8 mention disease stage or node involvement, and the study analyzed squamous cell carcinoma  
9 and adenocarcinoma histology together which may modify the survival.<sup>26</sup>  
10 Our study shows that molecular screening leads to an increased detection of MPLA compared  
11 to previous classifications mainly relying on histology, and could help to better classify  
12 patients with multiple lung tumors. When tumors-paired are classified as IPM in absence of  
13 lymph node metastasis, thus T3N0 or T4N0 according to the location, patients should receive  
14 an adjuvant treatment which is not the case when tumors are classified as MPLA tumors (T1-  
15 2N0). But large prospective studies are needed to assess the clinical value of this  
16 classification.

17

## 18 **Conclusion**

19 Our results showed that in standard care settings, our combined histo-molecular algorithm  
20 allows classification of multiple lung adenocarcinomas as IPM or MPLA. The absence of  
21 significant survival impact suggests that surgery is probably the best option when possible,  
22 even in oligo-metastatic situation. Classification could find its major application in post-  
23 surgical adjuvant treatment decisions with the assumption that some MPLA patients may not  
24 benefit from adjuvant chemo-radiotherapy and that some IPM patients may benefit from  
25 surgery and adjuvant therapy.

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1 **Author contributions:**

2 H.B. and D. D. are guarantors of the article.

3 H.B. and D. D. contributed to conception, design and leading the work;

4 A. M.-L., M. B., H. B., D. D., J. B. and M. A. contributed to data analysis and interpretation;

5 A. M.-L., M. B., D. D. and H. B. contributed to the drafting of the manuscript and to article  
6 redaction;

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9 All authors approved the final manuscript.

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## 1 Tables

	Variable	N (%)
Patients (N=120)	Sex	
	Female	49 (41%)
	Male	71 (59%)
	Age at first resection, mean [range]	63.5 [40-85]
	Smoking history	
	No	12 (10%)
	Yes	105 (87%)
	Unknown	3 (3%)
	Number of patients with different tumor chronology	
	Synchronous	86 (72%)
	Metachronous	34 (28%)
	Interval between resections, (months) mean [range]	
	Synchronous tumors	0.3 [0-10.9]
	Metachronous tumors	26 [6-80,3]
	Distribution of tumors	
Ipsilateral (same lobe)	50 (42%)	
Ipsilateral (other lobe)	45 (37%)	
Contralateral	25 (21%)	
Tumors (N=240)	Type of resection	
	Non-anatomic resection (wedge resection)	51 (21%)
	Segmentectomy	60 (25%)
	Lobectomy	100 (42%)
	Extended lobectomy (bilobectomy and pneumonectomy)	29 (12%)
	Side	
	Right	148 (62%)
	Left	92 (38%)
	Tumor size (cm) median [range]	1.8 [0.2-11]
	≤ 3	180
	3-5	41
	5-7	12
	> 7	7
	Adenocarcinoma subtype	
	Low grade	
	AIS	2 (<1%)
	MIA	10 (4%)
	Intermediate grade	
	Lepidic	7 (3%)
	Tubular	91 (38%)
	Papillary	59 (25%)
	High grade	
	Solid	48 (20%)
Mucinous	11 (4.5%)	
Cribriform	11 (4.5%)	
Micropapillary	1 (<1%)	
Nodal status		
N0	146 (60%)	
N1	30 (13%)	
N1 + N2	19 (8%)	
N2 without N1	15 (6%)	
Nx	30 (13%)	

1 Table 1: Clinical and histological characteristics of 120 patients operated for multiple lung  
2 adenocarcinomas.

3 Lung adenocarcinomas are classified into 3 histo-prognostic grades according to the 2015  
4 WHO classification. Metachronous tumors were defined by a second tumor occurring at least 6  
5 months after the first one. AIS: adenocarcinoma in situ; MIA: minimally invasive  
6 adenocarcinoma; Nx: regional lymph nodes not assessed.

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T	Histological pattern (predom.)	Molecular alterations	N	Martini class.	Chronology (m) and locations	Final class.	Surv. (m)
MPLA histology-IPM molecular (N=10)							
T1	MIA	KRAS p.G12C	N0		S		Alive
T2	Tubular	KRAS p.G12C	N0	MPLA	I (other lobe)	MPLA	(81)
T1	Papillary	EGFR p.L858R	N0		S		Alive
T2	tubular	EGFR p.L858R	N0	MPLA	C	MPLA	(79)
T1	solid	KRAS p.G12C // TP53 p.R337L	N0		S		Dead
T2	mucinous	KRAS p.G12C	N0	MPLA	I (other lobe)	MPLA	(1)
T1	papillary	KRAS p.G12V // CTNNB1 p.S37C	N0		S		Alive
T2	MIA	KRAS p.G12V	N0	MPLA	I (same lobe)	MPLA	(63)
T1	solid	KRAS p.G12C // TP53 p.G245C	N2		M (70)		Alive
T2	tubular	KRAS p.G12C // STK11 p.M51fs	Nx	MPLA	C	MPLA	(124)
T1	papillary	KRAS p.G12C	N0		S		Dead
T2	tubular	KRAS p.G12C	N0	IPM	I (same lobe)	MPLA	(58)
T1	solid (CC)	STK11 p.Y60* // ampl KRAS	N0		M (20)		Dead
T2	solid	STK11 p.Y60* // ampl KRAS	N1	IPM	I (other lobe)	IPM	(43)
T1	papillary	TP53 p.Y236C // PTEN c.635-99G>A // ampl ERBB2	N0		S		Dead
T2	solid	TP53 p.Y236C // PTEN c.635-99G>A	N1	IPM	I (same lobe)	IPM	(58)
T1	tubular	KRAS p.G12D // TP53 p.R273L	N0		M (13)		Dead
T2	solid	KRAS p.G12D // TP53 p.R273L // DDR2 p.E516Q	N1	IPM	I (same lobe)	IPM	(31)
T1	papillary	BRAF p.G469A // TP53 p.R273L // ERBB2 p.G882A	Nx		S		Alive
T2	solid	BRAF p.G469A // TP53 p.R273L	Nx	IPM	I (same lobe)	IPM	(51)
IPM histology-MPLA molecular (N =20)							
T1	tubular	KRAS p.G13C	Nx		S		Alive
T2	tubular	WT	Nx	MPLA	C	MPLA	(78)
T1	solid	STK11 p.M51fs // TP53 p.V157F // TP53 p.V272L	N0		M (20)		Alive
T2	solid	WT	Nx	MPLA	C	MPLA	(96)
T1	solid	NRAS p.Q61L	N0		M (62)		Alive
T2	solid	KRAS p.G12C // TP53 p.R249S	Nx	MPLA	I (other lobe)	MPLA	(139)
T1	solid (CC)	KRAS p.G13C	N0		M (52)		Alive
T2	tubular (CC)	KRAS p.G12C	Nx	MPLA	I (other lobe)	MPLA	(121)
T1	solid	TP53 p.E204*	N0		S		Dead
T2	solid	PIK3CA p.E545K // FBXW7 p.N592S	N0	MPLA	I (other lobe)	MPLA	(17)
T1	tubular	KRAS p.G12A // TP53 p.M237I	N0		S		Alive
T2	tubular	EGFR p.N771_P772insNV // TP53 p.G302fs	N0	MPLA	I (other lobe)	MPLA	(64)
T1	mucinous	KRAS p.G12V	N0		S		Alive
T2	mucinous	TP53 p.H214L	N0	MPLA	C	MPLA	(75)

T1	tubular	BRAF G466V // TP53 p.G154V // TP53 p.Y236C	Nx		S		Alive
T2	tubular	KRAS p.G12R // TP53 p.M246V	Nx	MPLA	I (other lobe)	MPLA	(60)
T3	papillary	KRAS p.G12C	N0		S		Alive
T4	papillary	KRAS p.Q61H	N0	MPLA	I (other lobe)	MPLA	(57)
T1	papillary	KRAS p.G12V	Nx		S		Alive
T2	papillary	KRAS p.G12C	Nx	MPLA	I (other lobe)	MPLA	(57)
T1	solid	KRAS p.G12V // STK11 p.P281fs	N0		S		Alive
T2	solid	BRAF p.V600E // ERBB4 p.G599W // TP53 p.G266V	N0	MPLA	I (other lobe)	MPLA	(51)
T1	tubular	ampl PIK3CA	N0		S		Dead
T2	tubular	KRAS p.G12D	N0	IPM	I (same lobe)	MPLA	(22)
T1	papillary	KRAS p.G12C	N1		S		Dead
T2	papillary	SMAD4 p.G352R	N1	IPM	I (other lobe)	MPLA	(7)
T1	solid	KRAS p.G12A	N2		S		Dead
T2	solid	WT	N2	IPM	I (other lobe)	MPLA	(2)
T1	tubular	TP53 p.D281Y	Nx		S		Dead
T2	tubular	TP53 p.H179L	Nx	IPM	I (same lobe)	MPLA	(17)
T1	papillary	KRAS p.G12V	N0		S		Alive
T2	papillary	STK11 p.Y49_L50fs	N0	IPM	I (same lobe)	MPLA	(76)
T1	tubular	KRAS p.G12C	N1		M (29)		Dead
T2	tubular	KRAS p.G12A	N1	IPM	C	MPLA	(50)
T1	tubular	EGFR p.K745_A750del // CTNNB1 p.S37C	N1		S		Alive
T2	tubular	EGFR p.L858R	N1	IPM	I (other lobe)	MPLA	(33)
T1	papillary	KRAS p.G12V // DDR2 p.R105C	N0		M (19)		Alive
T2	papillary	WT	N1	IPM	C	MPLA	(55)
T1	papillary	TP53 p.G245V	N2		S		Dead
T2	papillary	TP53 p.G105V // KRAS p.G12C // SMAD4 p.E193D	N2	IPM	I (other lobe)	MPLA	(33)

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2 Table 2: Final classification of the 30 discordant patients according to the proposed algorithm  
3 (Figure 1). Ampl: amplification; C: contralateral; CC: clear cells; class: classification; fs:  
4 frameshift; IPM: intra pulmonary metastasis; MPLA: multiple primary lung adenocarcinomas;  
5 MIA: minimally invasive adenocarcinoma; M: metachronous (months), m: month; N: Nodal  
6 status; predom: predominant; S: synchronous; Surv: survival; Tumor locations (I: ipsilateral,  
7 C:contralateral); WT: wild type.

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Variables	IPM (N=33)	MPLA (N=78)	p-value
Sex			
Female	12	36	0.4
Male	21	42	
Age at first resection, median [range] (years)	64 [40-82]	62 [45-85]	0.7
Smoking history			
Yes	24	73	<b>0.007</b>
No	9	5	
Tumors chronology			
Synchronous tumors	24	55	0.2
Metachronous tumors (< 5 years)	9	16	
Metachronous tumors (> 5 years)	0	7	
Interval between resections, median [range] (month)	21.1 [5.9-45.9]	35.7 [7.2-79.2]	<b>0.03</b>
Type of resection			
Sublobar resection (both tumors)	11	29	0.7
Supralobar resection (at least one tumor)	22	49	
Side			
Ipsilateral (same lobe) tumors	23	22	<b>0.0001</b>
Ipsilateral (other lobe) tumors	8	32	
Contralateral tumors	2	24	
Tumor size median [range] (cm)	2 [0.1-11]	1.6 [0.1-10]	0.07
Tumor grade			
Low or intermediate grade (both tumors)	21	46	0.8
High grade (at least one tumor)	12	32	
Node involvement			
N0 or Nx (both tumors)	17	57	<b>0.03</b>
N+ (at least one tumor)	16	21	
Pleural invasion			
No (both tumors)	11	36	0.2
Yes (at least one tumor)	22	42	
Vascular involvement			
No (both tumors)	5	26	0.09
Yes (at least one tumor)	28	52	
<i>EGFR</i> status			
WT (both tumors)	23	69	<b>0.03</b>
Mut (at least one tumor)	10	9	
<i>KRAS</i> status			



WT (both tumors)	24	24	<b>0.00005</b>
Mut (at least one tumor)	9	54	

1  
2 Table 3: Clinical, pathologic and molecular characteristics of 111 patients with multiple lung  
3 adenocarcinomas based on final classification (histo-molecular). Among the 120 patients  
4 included, 11 were wild type on both tumors and among the later 2 were finally reclassified as  
5 MPLA since one of their tumors was a minimally invasive adenocarcinoma), MPLA: multiple  
6 primary lung adenocarcinomas; IPM: intra pulmonary metastasis. Significant p-value < 0.05 are  
7 highlighted in bold.

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Variable	Univariate analysis			Multivariate analysis	
	estimate of survival at 5 years [95% CI]	p-value	HR [95% CI]	p-value	HR [95% CI]
Age at first resection *		0.08	1 [1-1.1]		
Smoking history					
No	71% [51%-99%]	0.1	2.1 [0.8-5.8]		
Yes	55% [46%-66%]				
Tumors chronology					
Metachronous	66% [52%-84%]	0.3	1.4 [0.8-2.5]		
Synchronous	54% [44%-66%]				
Interval between resections *		0.08	0.9 [0.7-1]		
Location					
Different lobes	63% [53%-75%]	0.2	1.4 [0.8-2.5]		
Same lobe	47% [34%-65%]				
Patient's largest tumor size *		0.6	1 [0.98-1]		
Grade status					
Low or Intermediate Grade (both tumors)	62% [51%-75%]	0.1	1.5 [0.9-2.6]		
High Grade (at least one tumor)	50% [37%-67%]				
Nodal status					
N0 or Nx	69% [59%-80%]	<b>0.0001</b>	<b>2.7 [1.6-4.5]</b>	<b>0.004</b>	<b>2.5 [1.4-5]</b>
N+	36% [24%-54%]				
Pleural invasion					
No (both tumors)	61% [49%-77%]	0.5	1.2 [0.7-2.1]		
Yes (at least one tumor)	54% [43%-67%]				
Vascular involvement					
No (both tumors)	76% [62%-93%]	<b>0.008</b>	<b>2.5 [1.2-5.2]</b>	0.2	1.8 [0.8-3.9]
Yes (at least one tumor)	50% [40%-62%]				
EGFR status					
WT (both tumors)	52% [43%-63%]	<b>0.03</b>	<b>0.3 [0.1-0.9]</b>	<b>0.02</b>	<b>0.3 [0.1-0.8]</b>
Mut (at least one tumor)	83% [67%-100%]				
KRAS status					
WT (both tumors)	58% [46%-73%]	0.9	1 [0.6-1.6]		
Mut (at least one tumor)	57% [45%-71%]				
TP53 status					
WT (both tumors)	55% [44%-69%]	0.4	0.8 [0.5-1.3]		
Mut (at least one tumor)	60% [48%-75%]				
Histological classification					
MPLA	59% [48%-72%]	0.9	1 [0.6-1.8]		
IPM	56% [44%-71%]				
Martini classification					
MPLA	70% [59%-82%]	<b>0.01</b>	<b>2 [1.1-3.4]</b>		
IPM	43% [32%-59%]				
Molecular classification					
MPLA	61% [51%-74%]	1	1 [0.6-1.8]		
IPM	52% [38%-71%]				
Final classification					
MPLA	62% [51%-74%]	0.5	1.3 [0.7-2.2]		
IPM	50% [35%-71%]				

1  
2 Table 4: Five years overall survival, univariate and multivariate analyses. For discrete variables a  
3 Kaplan-Meier method with a log-rank test was performed; for continuous variables\* a cox

1 proportional-hazards model with Wald-test was performed. Hazard ratios (HR) with 95%  
2 confidence interval (CI) were obtained using a Cox Model. Significant p-value < 0.05 and their  
3 HR are highlighted in bold.

4

5

## 1 **Figures legends**

2

3 Figure 1: 1A: Histological algorithm for classifying multiple lung adenocarcinomas into MPLA  
4 or IPM, based on 2015 WHO classification. This comprehensive histologic assessment involves  
5 evaluation of relative percentage of each architectural pattern defining predominant and minor  
6 patterns, cytologic and stromal features and TTF1 expression.

7 1B: Proposed algorithm for classifying multiple lung adenocarcinomas, based on histo-molecular  
8 criteria. AIS: adenocarcinoma in situ; MIA: minimally invasive adenocarcinoma; MPLA:  
9 multiple primary lung adenocarcinomas; IPM: intra pulmonary metastasis. Rare mutations are  
10 defined as non-*EGFR* (del 19 or p.L858R) and non-*KRAS* (p.G12X).

11

12 Figure 2: Contingency table for the comparison of histological and molecular classifications of  
13 tumors. Eleven tumors out of 120 are not included because they are not conclusive for molecular  
14 classification (both tumors were wild type). An illustration for each situation is shown.

15 The Kappa test ( $K = 0.43$ ) shows a fair agreement between both molecular and histological  
16 classifications.

17

18 Supplementary-Figure 1: Global distribution of gene mutations in the 240 studied lung  
19 adenocarcinomas.

20

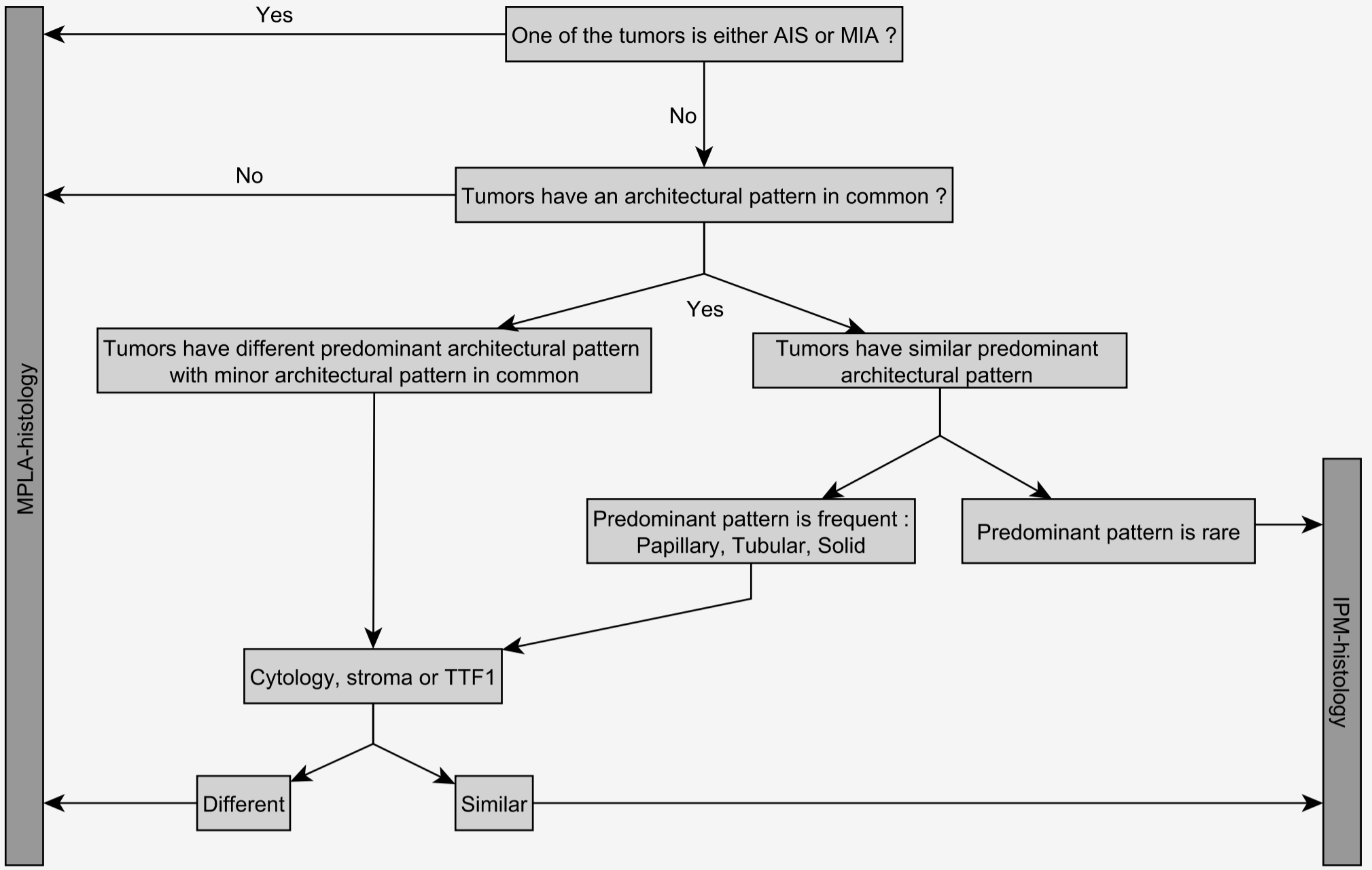
21 Supplementary-Figure 2: Molecular classification of patients with multiple lung tumors  
22 according to gene mutations. Both tumors (T1 and T2) are classified into 3 groups (MPLA, IPM  
23 and IPM w AA). MPLA: multiple lung primary adenocarcinoma is defined by the absence of  
24 common molecular mutation between both tumors (T1 and T2), or different driver mutations in  
25 *EGFR*, *KRAS*, *BRAF*, *ERBB2*, *NRAS* or *MET* genes. Intrapulmonary metastasis (IPM) is defined  
26 by a common molecular profile. Intrapulmonary metastasis with additional alterations (IMP w  
27 AA) is defined by at least one common alteration(s) with additional alteration(s) in one of the  
28 samples, including amplification. U: undetermined; Amp: amplification; Mut: mutation.

29

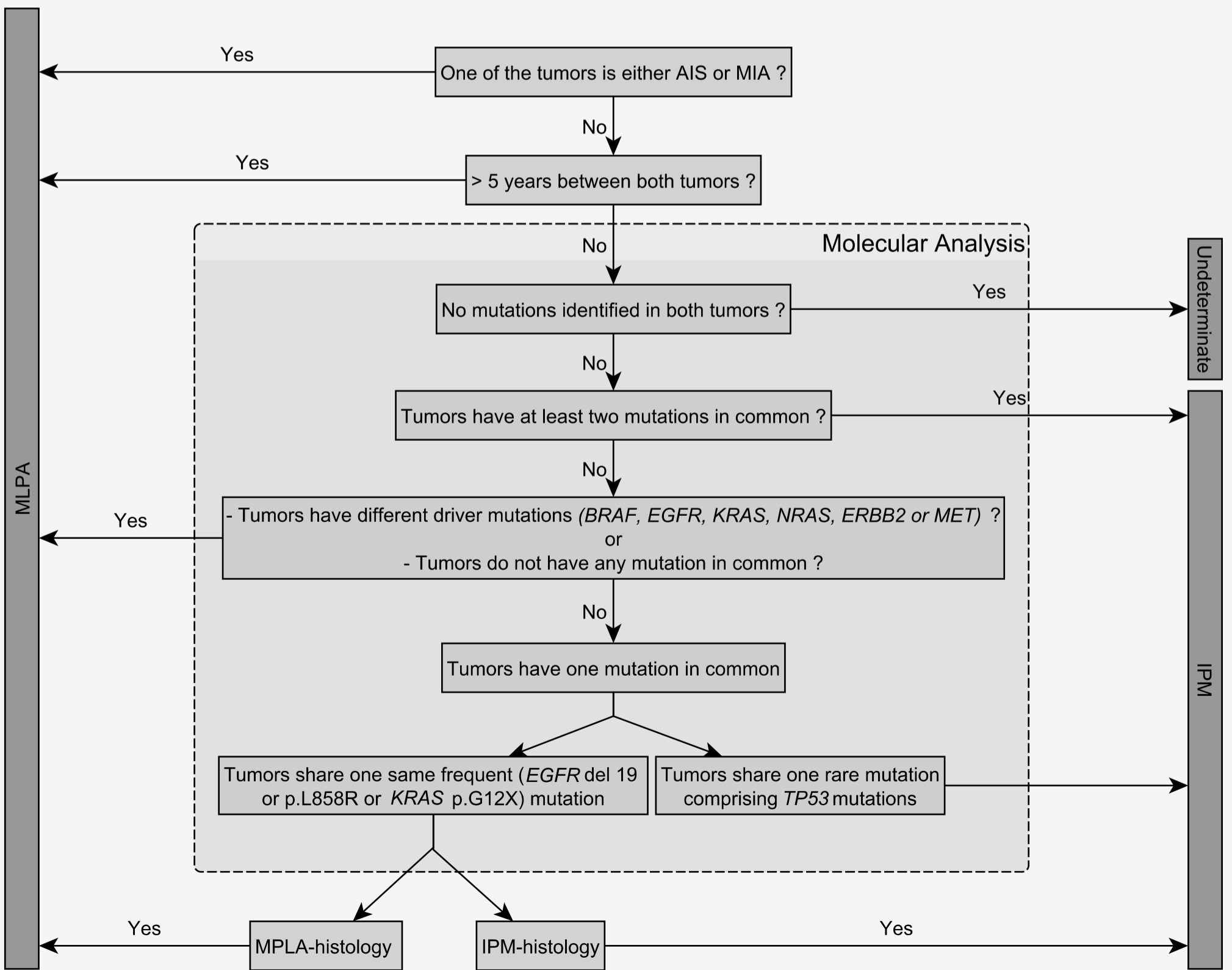
30 Supplementary-Figure 3: Repartition of *TP53*, *KRAS* and *EGFR* mutations according to MPLA  
31 and IPM final classification (histo-molecular) for the 111 patients.

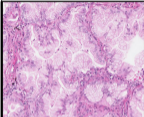
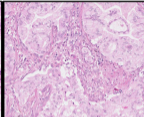
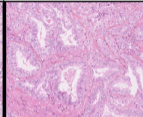
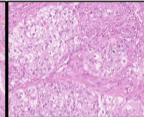
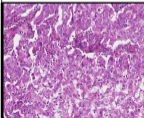
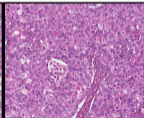
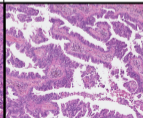
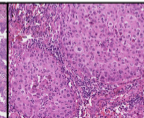
1  
2 Supplementary-Figure 4: Overall survival by Kaplan-Meier curves (log-rank test) of patients with  
3 multiple lung adenocarcinomas, according to EGFR status (A) and to Nodal-status (B). Survival  
4 was calculated from the resection of the first tumor.

## A/ Histological algorithm for classifying multiple lung adenocarcinomas



## B/ Integrated histo-molecular algorithm for classifying multiple lung adenocarcinomas



		Histological Classification			
		IPM : 49 patients		MPLA : 60 patients	
Molecular Classification	IPM : 39 patients	<b>29 patients</b>		<b>10 patients</b>	
		Mucinous	Mucinous	Tubular	Solid
					
		KRAS p.G12V	KRAS p.G12V	KRAS p.G12D TP53 p.R273L	KRAS p.G12D TP53 p.R273L DDR2 p.E516Q
Molecular Classification	MPLA : 70 patients	<b>20 patients</b>		<b>50 patients</b>	
		Solid	Solid	Papillary	Solid
					
		KRAS p.G12V STK11 p.P281fs	BRAF p.V600E ERBB4 p.G599W TP53 p.G266V	TP53 c.673-1G>T	TP53 p.C135W