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RESEARCH ARTICLE

InterMEL: An international biorepository and clinical database to uncover predictors of survival in early-stage melanoma

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Abstract

Introduction

We are conducting a multicenter study to identify classifiers predictive of disease-specific survival in patients with primary melanomas. Here we delineate the unique aspects, challenges, and best practices for optimizing a study of generally small-sized pigmented tumor samples including primary melanomas of at least 1.05mm from AJTCC TNM stage IIA-IIID patients. We also evaluated tissue-derived predictors of extracted nucleic acids' quality and success in downstream testing. This ongoing study will target 1,000 melanomas within the international *InterMEL* consortium.

Methods

Following a pre-established protocol, participating centers ship formalin-fixed paraffin embedded (FFPE) tissue sections to Memorial Sloan Kettering Cancer Center for the centralized handling, dermatopathology review and histology-guided coextraction of RNA and DNA. Samples are distributed for evaluation of somatic mutations using next gen sequencing (NGS) with the MSK-IMPACT™ assay, methylation-profiling (Infinium MethylationEPIC arrays), and miRNA expression (Nanostring nCounter Human v3 miRNA Expression Assay).

Results

Sufficient material was obtained for screening of miRNA expression in 683/685 (99%) eligible melanomas, methylation in 467 (68%), and somatic mutations in 560 (82%). In 446/685 (65%) cases, aliquots of RNA/DNA were sufficient for testing with all three platforms. Among samples evaluated by the time of this analysis, the mean NGS coverage was 249x, 59 (18.6%) samples had coverage below 100x, and 41/414 (10%) failed methylation QC due to low intensity probes or insufficient Meta-Mixed Interquartile (BMIQ)- and single

sample (ss)- Noob normalizations. Six of 683 RNAs (1%) failed Nanostring QC due to the
low proportion of probes above the minimum threshold. Age of the FFPE tissue blocks
(p<0.001) and time elapsed from sectioning to co-extraction (p = 0.002) were associated
with methylation screening failures. Melanin reduced the ability to amplify fragments of
200bp or greater (absent/lightly pigmented vs heavily pigmented, p<0.003). Conversely,
heavily pigmented tumors rendered greater amounts of RNA (p<0.001), and of RNA above
200 nucleotides (p<0.001).

Conclusion

Our experience with many archival tissues demonstrates that with careful management of
tissue processing and quality control it is possible to conduct multi-omic studies in a complex
multi-institutional setting for investigations involving minute quantities of FFPE tumors, as in
studies of early-stage melanoma. The study describes, for the first time, the optimal strategy
for obtaining archival and limited tumor tissue, the characteristics of the nucleic acids co-
extracted from a unique cell lysate, and success rate in downstream applications. In addi-
tion, our findings provide an estimate of the anticipated attrition that will guide other large
multicenter research and consortia.

Introduction

Melanoma accounts for the great majority of deaths from skin cancer with an estimated 7,650
deaths in the USA in 2022 [1]. The five-year survival rate of melanoma ranges from 98% for
localized disease to less than 30% in patients with distant metastases at the time of diagnosis
[2,3]. Factors known to affect progression and survival include primary tumor characteristics
[4,5], presence of nodal or distant metastases at the time of diagnosis [6], anatomic site of the
tumor [7], as well as demographic characteristics such as age at diagnosis [8,9] and sex [9];
however, the prediction of individual outcomes based on clinicopathologic factors has limited
power. The addition of molecular characteristics has the potential to improve risk classification
and help identify individuals more likely to benefit from a differential surveillance schedule
and/or therapies.

To date, the most comprehensive efforts undertaken to reveal the multifaceted molecular
characteristics of cutaneous melanoma were reported by The Cancer Genome Atlas (TCGA)
[10]. These investigations led to the classification of melanomas into four subtypes based on
somatic *BRAF*, *RAS*, *NF1* mutations, and triple wild-type, and the identification of phenotypic
characteristics (i.e., tumor infiltrating lymphocytes and LCK protein expression) linked to
improved survival. The number of primary melanomas in TCGA, however, was small (n~67)
and the thickness of these tumors was larger than the vast majority of primary melanomas,
which are usually very thin at diagnosis. Thus, findings may not be generalizable. To be able to
translate research findings into the real clinical and diagnostic practice, it is necessary to utilize
melanoma tissue that remains available once histopathologic diagnosis is completed as part of
the standard care. Therefore, for findings to be generalizable, investigations need to focus on
the use of limited archival tissue from small melanomas.

In this article we describe our experiences organizing the tissue collection and processing of
specimens that are being collected by the multi-institutional InterMEL Consortium [11], a
study designed to define molecular subtypes of cutaneous primary melanoma of at least 1.05

mm in patients diagnosed with AJCC TNM stages IIA through IIID melanoma to improve the prognostic stratification, to guide enhanced surveillance, and/or the use of adjuvant therapies for early intervention, as necessary.

The use of targeted and immuno-based therapies for stage III melanoma patients has been shown to be effective in improving recurrence-free survival [12]. Also, pembrolizumab has just received FDA approval as adjuvant treatment for stage IIB/C melanoma based on its significant efficacy compared to placebo in preventing both locoregional and distant recurrence in a double-blind clinical trial [13]. Therefore, it is pertinent and timely to better identify which patients are at highest risk who would most likely benefit from these treatments and which patients are at lower risk who should be spared of unnecessary, potentially toxic, and costly treatments [14].

The InterMEL Consortium is collecting clinical and pathologic data, as well as archived melanoma tissues, to screen for somatic mutations and evaluate miRNA and mRNA expression, methylation, protein expression, and to then correlate these features with survival. A uniquely challenging aspect of the current study is the use of archived primary melanomas, which are typically of limited size. To address this concern our study design involves the systematic co-extraction of DNA and RNA from the same tissue sections and derived cell lysates. This study design allows us to save tissue, and most importantly, it allows us analysis of molecular data from the same portion of tumor, eliminating potential biases due to intratumor heterogeneity.

Our objectives were to develop the best practices for optimizing a large multicenter study of generally small-sized archival pigmented tumor samples obtained from early-stage melanoma patients, and to share our step-by-step approach, the unique aspects and challenges, and biospecimens characteristics that may predict success in downstream testing. In this report we detail procedures involved in establishing the appropriate infrastructure for our multicenter, integrated study. These include tissues and workflow, sample quality and quantity parameters, and criteria for DNA and RNA distribution for testing, for procuring a balanced proportion of controls (survivors for >5 years with no evidence of disease) and cases (dead within 5-years of follow up) across tests. We also report on the various quality control (QC) parameters used. Based on our experience on already procured data and FFPE tissues from 685 eligible early-stage melanomas from 10 centers, we provide insights into the logistic and technical challenges involved when conducting a large patho-epidemiologic international and multicenter study of disease-specific survival in 1,000 primary melanomas from stage II/ III patients.

Methods

Overview

This retrospective ongoing study aims to collect biospecimens and data from a total of 1,000 eligible melanoma patients, including patients who died of melanoma within 5 years of diagnosis (referred to as cases) and patients who lived/are alive for at least 5 years after diagnosis, without evidence of regional or distant melanoma recurrence or relapse (referred to as controls). Participants are identified through the multi-center and international consortium 'Integration of Clinical and Molecular Biomarkers for Melanoma Survival' or InterMEL [11], as further detailed below. The study protocol was approved by the Institutional Review Boards (ethics committees) at each participating institution, material and data user agreements are in place, and research has been conducted according to the principles expressed in the Declaration of Helsinki. The need for informed consent was waived by the ethics committees due to the retrospective nature of the study. The Biospecimen Core provides complete pathology and

biospecimens support, and appropriate infrastructure to all investigations. Activities are detailed next and in the Supporting Information.

Eligibility criteria

For cases and controls:

- Diagnosis of primary cutaneous melanoma on or after January 1, 1998 and prior to January 1, 2016

- Stage IIA-IIID re-staged according to the AJCC 8th edition

- Melanoma tumor thickness >1.05 mm, using the rounding metrics in the AJCC 8th edition

- If melanoma thickness is 1.00 to 2.00 mm and stage is IIA, tumors should be ulcerated.

- Index tumor must be a first primary invasive melanoma (i.e. no previous invasive melanomas; melanoma-in-situ is okay.)

- Have sufficient tissue available for providing tissues as specified by the Tissue Handling and Sectioning Protocol:

○ Seven 10 μm sections on uncharged slides, unstained, uncharged), plus

○ two 4 or 5 μm sections placed on charged slides, unstained, and

○ two H&E, one representing the beginning and one the end of the sectioning

- Patient has not received adjuvant immunotherapy or targeted therapy (e.g. vemurafenib, dabrafenib, cobimetineb, trametinib, ipilimumab, nivolumab, pembrolizumab). Interferon and IL2 are allowed.

  Specific for cases:

- Patient died of melanoma within 5 years of initial diagnosis

  Specific for controls:

- Patient alive and without evidence of melanoma regional or distant recurrence or relapse for at least 5 years after diagnosis. Participants with a clear recurrence at the positive edge of the margins are eligible.

  For cases and controls: if eligible according to the above criteria, patients treated with immune- or targeted therapy when reaching stage IV, remain eligible.

Participants

For each participant, the following data elements are being collected by the participating study centers: demographics, vital status, year of diagnosis, follow up time, progression, recurrence, anthropomorphic measures, treatment type. For the index primary tumors, single *versus* multiple status, TNM stage (AJCC Ed. 8), tumor burden or load, and anatomic site are also recorded. The following study centers provided patients' data and biospecimens as of March 7th 2021: Cleveland Clinic (Cleveland, OH), Case Western Reserve University (Cleveland, OH), Dartmouth Cancer Center (Lebanon, NH), the University of Texas MD Anderson Cancer Center (Houston, TX), Melanoma Institute Australia (Sydney, NSW, Australia), Memorial Sloan Kettering Cancer Center (New York, NY), New York University Langone Medical Center (New York, NY), The University of North Carolina (Chapel Hill, NC, hereafter denoted UNC), Roswell Park Comprehensive Cancer Center (Buffalo, NY),Yale School of Medicine

(New Haven, CT). Additional centers are currently preparing data and tissue for participants not included in this report.

Biospecimens

The study collects archived tumor and counterpart non-tumor tissue (or germline DNA), following a detailed protocol and shipping manifest template for the assembly, labeling, transport and shipping of samples, and communication procedures that are provided in advance to each participating center, with further guidance provided as needed by the Biospecimens and the Administrative Cores. All tumor samples are procured from FFPE tissue blocks sectioned at each of the contributing centers, for this study.

The overall flow of specimens and related information is depicted in Fig 1. First, study centers provide the minimal electronic data required to verify eligibility of cases and controls centrally (T.K.L.), and then eligibility is communicated to the centers, and to the Biospecimens
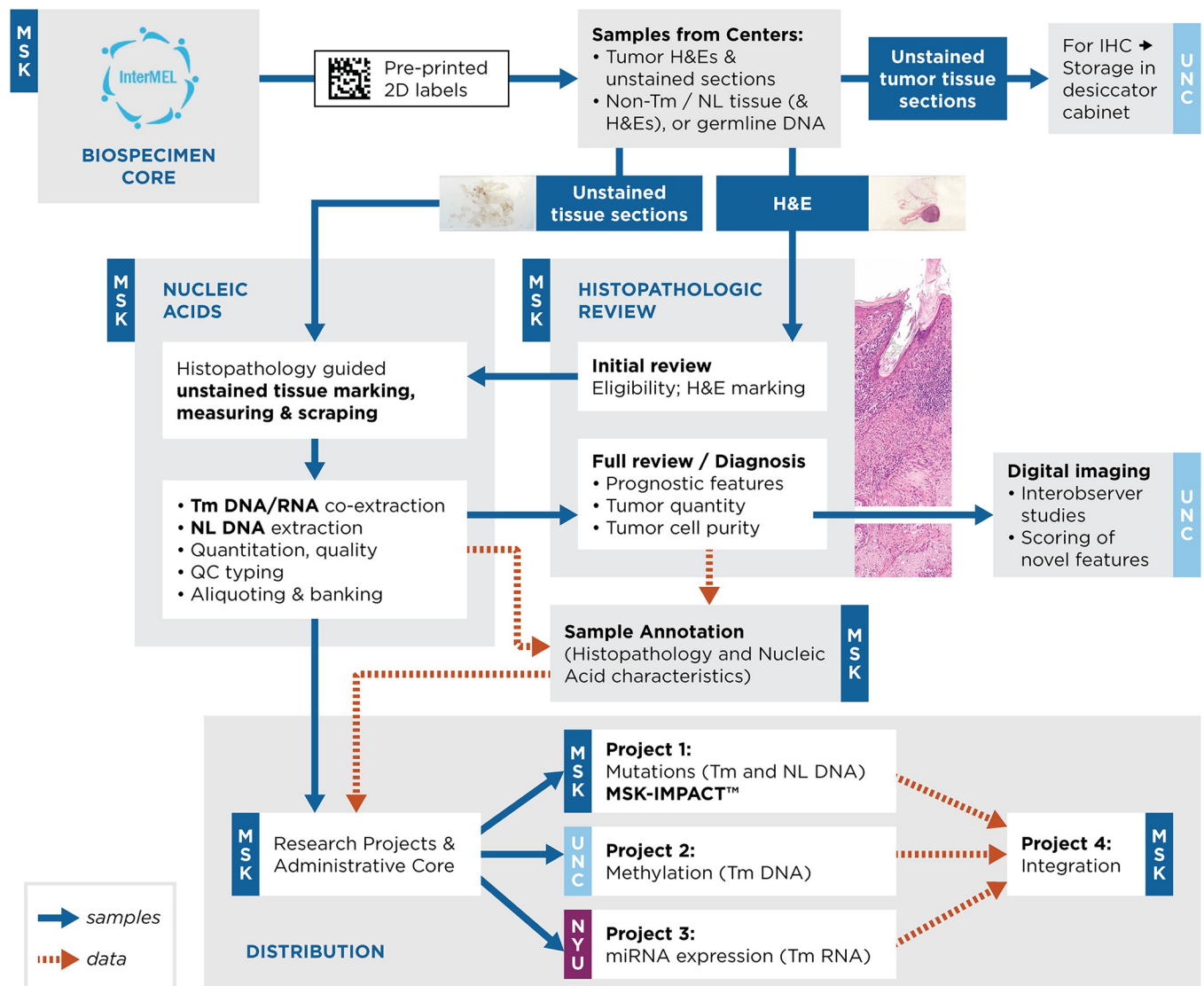

Fig 1. Summary of specimens and data flow in InterMEL.

and Administrative Cores. For eligible melanomas, members of the Molecular Epidemiology Laboratory at Memorial Sloan Kettering (MSK) in New York print and mail 2D alphanumeric temperature and solvent resistant labels to the centers. The study centers, in turn, prepare tumor and non-tumor samples following a common Biospecimen Core Tissue Handling and Sectioning Standard Operating Procedure. Detailed procedures and recommendations are provided in the S1 Appendix.

Pathology review

Tumor H&Es undergo two rounds of centralized pathology review by expert dermatopathologists at MSK (K.B., C.L.). The initial pathology review serves to confirm the eligibility of the case and the eligibility of the tissue for nucleic acid extractions. The eligible area is marked with a water-resistant pen directly on the H&E. The eligible and marked H&Es are then utilized in the Laboratory as guides for (a) determining and annotating the size of the qualifying tissue area, and (b) for marking the unstained tissue sections in preparation of nucleic acid extraction (see below). Next, the tumor H&Es are returned to the reference pathologist for a second–more in-depth, or 'full'- pathology review. Here, melanoma diagnosis, histopathologic prognostic features, volume, viability, and purity of available tumor tissue are documented. The annotated histopathologic prognostic features included, at minimum: Breslow thickness, ulceration, melanoma subtype (ICD-0-3 morphology code), Clark level, mitotic rate, tumor infiltrating lymphocytes (TIL) grade, regression, pigmentation, associated nevus, solar elastosis, predominant cell type, neurotropism. Additional salient observations (e.g., whether tissues appeared to be decalcified, necrotic, or include scarring) are also noted. Participants and tumor variables and their definitions are listed in the (S1 Table).

Histopathology-guided co-extraction of nucleic acids from archival tissues

For each tumor specimen, systematic marking of qualifying unstained tissue areas is performed. This is followed by careful scraping of tissues and batched co-extraction of nucleic acids using the AllPrep® DNA/RNA FFPE Kit (Qiagen). Details on the preliminary optimization, co-extraction procedures, and aliquoting rules, can be found in the (S1 Appendix, S1 Fig in S3 Appendix, S2 Appendix). The extraction kits and procedures utilized for procurement of germline DNA (gDNA), and the detailed procedures utilized for the assessment of DNA and RNA quantity and quality, are also found in the (S1 Appendix). Upon completion of the co-extraction, H&Es are scanned (for future image analysis).

Criteria for allocation of specimens for testing

Primarily, we use the amount of double stranded (dsDNA) tumor and normal DNA, and total RNA as criteria for distribution for testing. To date, RNA samples with yields of 500ng or greater were distributed for testing. The distribution of DNA involves consideration of quality, quantity and availability of tumor and normal DNA for Next Generation Sequencing (NGS), and a balanced distribution of samples/cases for methylation and NGS. Specifically, we triage study material using the following rules:

a. When equal or greater than (≥) 350ng of tumor DNA and ≥100ng of normal DNA are obtained, DNAs are distributed for <u>both</u> mutation (tumor-normal paired DNAs) and methylation testing (tumor DNA).

b. When 250 to 349ng of tumor DNA and ≥ 100ng of normal DNA are available, DNA samples are distributed for <u>either</u> mutation or methylation testing, procuring a balanced distribution of controls (survivors) and cases (dead within 5-years of follow up) across tests.

c. Samples with less than 70ng of DNA were held back.

d. Participants that lack or have insufficient amounts of germline or non-tumor DNA available are held back until sent as a tumor DNA-only batch for NGS.

When pairing tumor and normal DNAs for NGS, we take into consideration both amounts and quality of the DNA. For example, relatively greater amounts of FFPE tumor DNA are paired with germline DNA extracted from blood (or lower amounts of blood-DNA is paired with tumor DNA). Qualifying specimens are plated and distributed with accompanying documentation for testing of mutations at MSK, methylation at UNC and miRNA profiling at the NYU.

Screening of somatic mutations, methylation, and miRNA expression

The methodology is described in detail in the Supporting Information, within the S1 Appendix.

Briefly, DNA samples are sequenced at Memorial Sloan Kettering using the Integrated Mutation Profiling of Actionable Cancer Targets or MSK-IMPACT™, a clinically validated and FDA approved hybridization capture-based next-generation sequencing assay developed to guide cancer treatment [15,16]. At the University of North Carolina, DNA samples undergo a bisulfite modification, and genome-wide DNA methylation analysis is performed using the Infinium MethylationEPIC BeadChip kit (Illumina). Filtering and normalization of output methylation data obtained from the Infinium MethylationEPIC arrays is done in R [17]. RNA samples are evaluated for miRNA expression at New York University using the Nanostring nCounter® Human v3 miRNA Expression Assay, which includes 800 microRNAs.

Data handling

The centers providing melanoma specimens prepare an Excel file containing the de-identified information necessary to assess eligibility. This file is uploaded onto a secure cloud where information is checked semi-automatically assisted by a custom-built SAS program. As of March 7th, 2021, 793 Excel records (325 cases and 468 controls) were assessed, and 296 (92%) cases and 413 (88%) controls were deemed eligible for the study. Additional information regarding communication of eligibility, annotation of data, and data sharing, is provided in the (S1 Appendix).

Statistical analysis

Relationships between continuous variables (e.g., tissue area and DNA yield) were tested using Pearson correlation tests. Fisher's Exact test was used to evaluate the associations among categorical variables (e.g., survival group and ulceration), and Welch t-test and ANOVA were used to evaluate potential associations between continuous and categorical variables. Tumor purity expressed by the proportion of tumor cells, was dichotomized into >0.7 or ≤ 0.7 (>70 and $\leq 70\%$ tumor cells). DNA amplifiability was dichotomized two ways, according to the DNA amplification of two or more fragments, and three or more fragments. Pigmentation (absent, lightly pigmented, or heavily pigmented) and the IGO core DNA and Library QC recommendations (fail, try, pass) were analyzed as three categories, or collapsed into two categories: pigmentation absent/lightly pigmented vs heavily pigmented, and recommendations Fail vs Try/Pass. Associations were illustrated using scatterplots, boxplots, and bar graphs. Statistical analyses were performed in R [17], v.3.6.3.

Results

Study participants and melanomas

This report includes the data and analysis for 685/718 eligible melanomas received at MSK as of March 7th, 2021. Upon completion of the initial central pathology review, tissue ineligibility was attributed to:

- Inability to confirm a primary melanoma diagnosis on tissue sections provided for centralized review: lymph node metastasis with no definitive primary melanoma; absence of visible skin; presence of mostly congenital, residual, or deep penetrating nevus

- Inadequate tissue quality (e.g., decalcified, mostly scar, or mostly necrotic); and/or quantity (e.g., melanoma size and/or amounts of remaining tumor

Normal tissue was excluded from further analysis when inadequate in size, contaminated with tumor, or when the counterpart tumor was deemed ineligible. Subsequently, 9 confirmed melanoma patients were also excluded because they were lost to follow up and/or had died from unknown causes.

A second centralized and full pathology review was completed in 561/685 melanomas (as of the cut-off date for the current analysis and manuscript), and data are summarized in Table 1. Of these 327 (58.3%) are "controls" (survived >5 years without a melanoma recurrence) and 234 (41.7%) are "cases" (died within the 5-year follow up period). Most participants were reported being male (61.5%), white (95.2%) and had a median age at diagnosis of 64 years. Tumor thickness ranged from 1.1 to 120mm. Two-hundred and eighty-three of the participants (52.2%) presented at diagnosis with stage II and 268 (47.8%) with stage III at diagnosis. Additional variables of interest were captured (Table 1).

Co-extracted tumor RNA and DNA, and gDNA

Results from the preliminary assessment of two co-extraction kits done through an independent, pilot set of FFPE tissues include quantity and quality assessment, as well as genetic and epigenetic testing (S2 Appendix, S1 and S2 Figs in S3 Appendix). For the current report and ongoing study, the characteristics of the FFPE target tissue included in the RNA-DNA co-extraction (tumor FFPE tissue) and in the DNA extraction (non-tumor FFPE tissue) are shown on Table 2. The total amount of RNA and dsDNA obtained per case increased with the size of the target tumor tissue, but this was not the case for the dsDNA obtained from the target non-tumor FFPE (S3 Fig in S3 Appendix). Quantity and quality characteristics of tumor and non-tumor DNA, and tumor RNA, are shown on Table 3. Inter-batch replicas had very similar miRNA expression profiles (correlation coefficient r>0.9), indicating stability of the miRNA in the sample. In general, no significant differences were noticed across batches of extracted tumors, or lots of reagents. Fingerprinting, testing with SampleID (Agena Bioscience), and/or NGS revealed six unresolvable discrepant cases for which new specimens were requested. In four of these, we found differences between the genetic and reported sex, and between tumor and paired non-tumor DNAs. Germline DNAs were extracted from 375 FFPE non-tumor tissues (279 sets of sections and 96 sets of curls), 15 blood samples, and one saliva sample.

Melanoma characteristics and quantity/quality of Nucleic Acids (NA)

The tumor characteristics significantly associated with the obtained NA quantity and/or quality are depicted on Fig 2 and S4 Fig in S3 Appendix. There was a positive correlation between the extracted target tissue area and the obtained DNA (Fig 2A) and RNA yield (Fig 2B). Greater quantities of RNA relative to the extracted tumor area were obtained among stage III

Table 1. Demographic, clinical, and detailed pathologic characteristics for patients and melanomas at the time of initial diagnosis (total, n = 561*).

Patient and tumor characteristics		N (proportion)
Survival group		
	Died within 5 years of diagnosis	234 (41.7%)
	Lived more than 5 years after diagnosis	327 (58.3%)
Center		
	CCF	87 (15.53%)
	CW	15 (2.7%)
	DTM	77 (13.7%)
	MDA	30 (5.3%)
	MIA	237 (42.2%)
	MSK	6 (1.1%)
	NYU	37 (6.6%)
	UNC	49 (8.7%)
	RPCI	1 (0.2%)
	Yale	22 (3.9%)
Year of diagnosis, median years (IQR)		
		2007 (2004–2009)
Sex		
	Male	345 (61.5%)
	Female	216 (38.5%)
Age at diagnosis, median years (IQR)		
		64 (52–75)
Race		
	White	534 (95.2%)
	Asian	6 (1.1%)
	Black or African American	5 (0.9%)
	Other	6 (1.1%)
	Missing	10 (1.8%)
Anatomic site		
	Head and Neck	129 (23%)
	Trunk	158 (28.1%)
	Arms	102 (18.2%)
	Legs	170 (30.3%)
	Skin, unspecified	2 (0.4%)
Recurrence		
	No	210 (37.4%)
	Yes	310 (55.3%)
	Missing	41 (7.3%)
Stage		
	II	293 (52.2%)
	III	268 (47.8%)
Pathologic stage		
	II	9 (1.6%)
	IIA	107 (19.1%)
	IIB	118 (21%)
	IIC	59 (10.5%)
	III	8 (1.4%)

(Continued)

Table 1. (Continued)

Patient and tumor characteristics		N (proportion)
	IIIA	30 (5.3%)
	IIIB	51 (9.1%)
	IIIC	155 (27.6%)
	IIID	24 (4.3%)
Breslow thickness, median mm (IQR)		
		3.8 (2.5–6.2)
Clark level		
	2	1 (0.2%)
	3	20 (3.6%)
	4	421 (75%)
	5	103 (18.4%)
	Cannot assess	13 (2.3%)
	Missing	3 (0.5%)
Ulceration		
	Absent	234 (41.7%)
	Present	326 (58.1%)
	Cannot assess	1 (0.18%)
Mitoses		
	Absent	37 (6.6%)
	Present	522 (93%)
	Missing	2 (0.4%)
Perineural invasion		
	Absent	453 (80.7%)
	Present	93 (16.6%)
	Cannot assess	15 (2.7%)
Histology		
	SSM	149 (26.6%)
	NM	61 (10.9%)
	LMM	26 (4.6%)
	ALM	28 (5%)
	Cannot assess	293 (52.2%)
	Missing	4 (0.7%)
Solar elastosis		
	Absent	250 (44.6%)
	Mild/Moderate	150 (26.7%)
	Severe	104 (18.5%)
	Cannot assess	57 (10.2%)
Associated nevus		
	Absent	488 (87%)
	Present	46 (8.2%)
	Cannot assess	24 (4.3%)
	Missing	3 (0.5%)
Pigmentation		
	Absent	224 (39.9%)
	Lightly pigmented	250 (44.6%)
	Heavily pigmented	84 (15%)
	Missing	3 (0.5%)

(*Continued*)

Table 1. (Continued)

Patient and tumor characteristics		N (proportion)
TILs		
	Brisk	22 (3.9%)
	Non-brisk	471 (84%)
	Absent	59 (10.5%)
	Cannot assess	7 (1.2%)
	Missing	2 (0.4%)

*N = 561, total number of eligible melanomas that underwent full pathology review at the time of the analyses.
Abbreviations: CW, Case Western Reserve University; CCF, The Cleveland Clinic Foundation; DTM, Dartmouth Cancer Center; MDA, The University of Texas M.D. Anderson Cancer Center; MIA, Melanoma Institute of Australia at The University of Sydney; MSK, Memorial Sloan Kettering Cancer Center; NYU, New York University; UNC, The University of North Carolina at Chapel Hill; UNM, University of New Mexico; RPCI, Roswell Park Comprehensive Cancer Center; Yale, Yale School of Medicine. IQR, interquartile range; TILs, tumor infiltrating lymphocytes.

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melanomas compared to those with stage II: 39.5 vs 32.4 ng/mm2 RNA (p<0.01); but for DNA this difference did not reach statistical significance (4.6 vs 3.9 ng/mm2 dsDNA, p = 0.06). No associations were observed between the quantity of co-extracted DNA/RNA and tumor Breslow thickness, tumor purity (% tumor cells), or time elapsed between tissue block sectioning to co-extraction. Time elapsed, however, had an inverse statistically significant association with amplicon size on QC-PCR (Fig 2C). TILs were significantly associated with greater amounts of dsDNA (Fig 2D) and purity (S4A Fig in S3 Appendix). Presence of TILs was significantly associated with the ability to generate amplicons >200bp (S4B Fig in S3 Appendix). There was a positive association between pigmentation and quantities of total RNA obtained, with heavily pigmented tumors rendering higher yields compared to both lightly pigmented and non-pigmented tumors (Fig 2E). DV200 values were also higher in RNAs extracted from pigmented lesions (Fig 2F). Ulceration was significantly associated with greater DNA A260/A280 (Fig 2G) and RNA A260/A280 values (p<0.045, not shown). DNA and RNA samples obtained from heavily pigmented tumors had lower A260/A280 ratios

Table 2. Assessment of target FFPE tissue areas for extractions of nucleic acids from tumor and non-tumor specimens.

	Tumor, N = 685^	Non-tumor, N = 279^*
Number of sections, median (range)	7 (4–25)	5 (1–15)
% tumor, median (range)	80 (15–95)	0 (0–40)
Pigmentation visible, N	44	13
Sections thickness, mm median (range)	10 (5–10)	10 (10–10)
Length, mm median (range)	10.5 (2–98)	18 (1–46)
Width, mm median (range)	3 (0.5–21)	6 (0.25–23)
Area per section, mm2 median (range)	36 (1.8–480)	98 (1–600)
Total tissue area mm2 median (range)	273 (12.6–4950)	510 (5–4725)
*Time elapsed, days (sectioning to extraction) median (range)	163 (15–655)	112(30–663)

^*Number of tissues extracted by the March 7th, 2021 cut-off (this report). *Counts do not include tumors (n = 2) and non-tumors (n = 94) for which tissue sections were provided as 'curls', saliva (n = 1), or blood/buffy coat (n = 209). Note that characteristics are for tumors which underwent initial central histopathology review. The targeted tissue refers to the histopathology-guided and marked tissue areas deemed suitable for being scraped off of glass slides in preparation of the extraction of nucleic acids.

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Table 3. Characteristics of RNA and DNA obtained from tumor and non-tumor tissues.

		RNA					
		Nanodrop			TapeStation		
Tumor	Total RNA (ng)	A260/A280	A260/A280	Total RNA (ng)	RIN	% >200nt	Total >200nt (ng)
FFPE, N = 685							
Average	11477.7	1.89	0.92	1967.9	2	43.8	1016.1
Median	6432.5	1.9	0.83	1203.3	2	44.1	523
Min	246	1.27	0.03	56.9	1	7.9	7.2
Max	201716	3	5.16	16989	4	100	11464.2
		DNA					
		Nanodrop		Qubit		QC PCR	
Tumor	Total DNA (ng)	A260/A280	A260/A230	dsDNA (ng)	%dsDNA	N bands	
FFPE, N = 685							
Average	5395.2	1.9	0.9	1283.4	23.9	2.1	
Median	3223.4	1.8	0.7	659.1	23.1	2	
Min	53.9	-1.9	-19.3	3.1	1	0	
Max	95515.3	9.1	28	25841.5	93.4	4	
		Nanodrop		Qubit		QC PCR	
Normal	Total DNA (ng)	A260/A280	A260/A230	dsDNA (ng)	%dsDNA	N bands	
FFPE, N = 279							
Average	13064.0	2.16	1.34	2467.4	19.8	2.2	
Median	5097.3	1.99	1.50	573.4	18.2	2.0	
Min	136.5	-1.70	0.02	1.2	0.6	0.0	
Max	155270.6	6.22	6.24	55316.0	172.5	4.0	
Blood, N = 9							
Average	8875.0	1.51	0.60	4945.5	46.8	3.4	
Median	7354.0	1.62	0.58	1310.8	32.3	4.0	
Min	1353.8	1.07	0.25	196.1	5.0	2.0	
Max	19888.1	1.85	1.22	17899.3	90.0	4.0	

(Fig 2H–2I). DNA from pigmented tumors amplified shorter fragments (S4C Fig in S3 Appendix). Stage III, tumor purity, and presence of ulceration were all positively associated with DNA A260/A230 ratios (p = 0.04, p = 0.04, and p = 0.004, respectively; S4D-S4F Fig in S3 Appendix). Evaluation of absorbance (260/280, and 260/230) ratios was limited to samples with concentrations ≥20ng/µL as lower concentrations render inaccurate data. Fig 3 depicts the effect of case characteristics (Fig 3A), time elapsed from sectioning to co-extraction (Fig 3B), and tumor characteristics (Fig 3C and 3D), on the recommendations to proceed with the synthesis of libraries. In addition, we observed a statistically significant association between presence of ulceration and DNA-QC 'failures' (p<0.045), with 15% of DNA samples categorized as failed among ulcerated melanomas compared to 28% failed DNAs among those without ulceration (p<0.001). The effect of DNA and RNA quality characteristics (purity, fragmentation/integrity) on the recommendations to proceed with (a) the synthesis of libraries and (b) MSK-IMPACT[TM] assay are shown in the (S5 Fig in S3 Appendix).

Distribution of nucleic acids for genetics and epigenetics testing, and preliminary QC

Almost all RNA samples, 683/685 (99%), qualified for distribution for miRNA expression analysis with the Nanostring nCounter® Human v3 miRNA Expression assay, 467 (68%) DNA
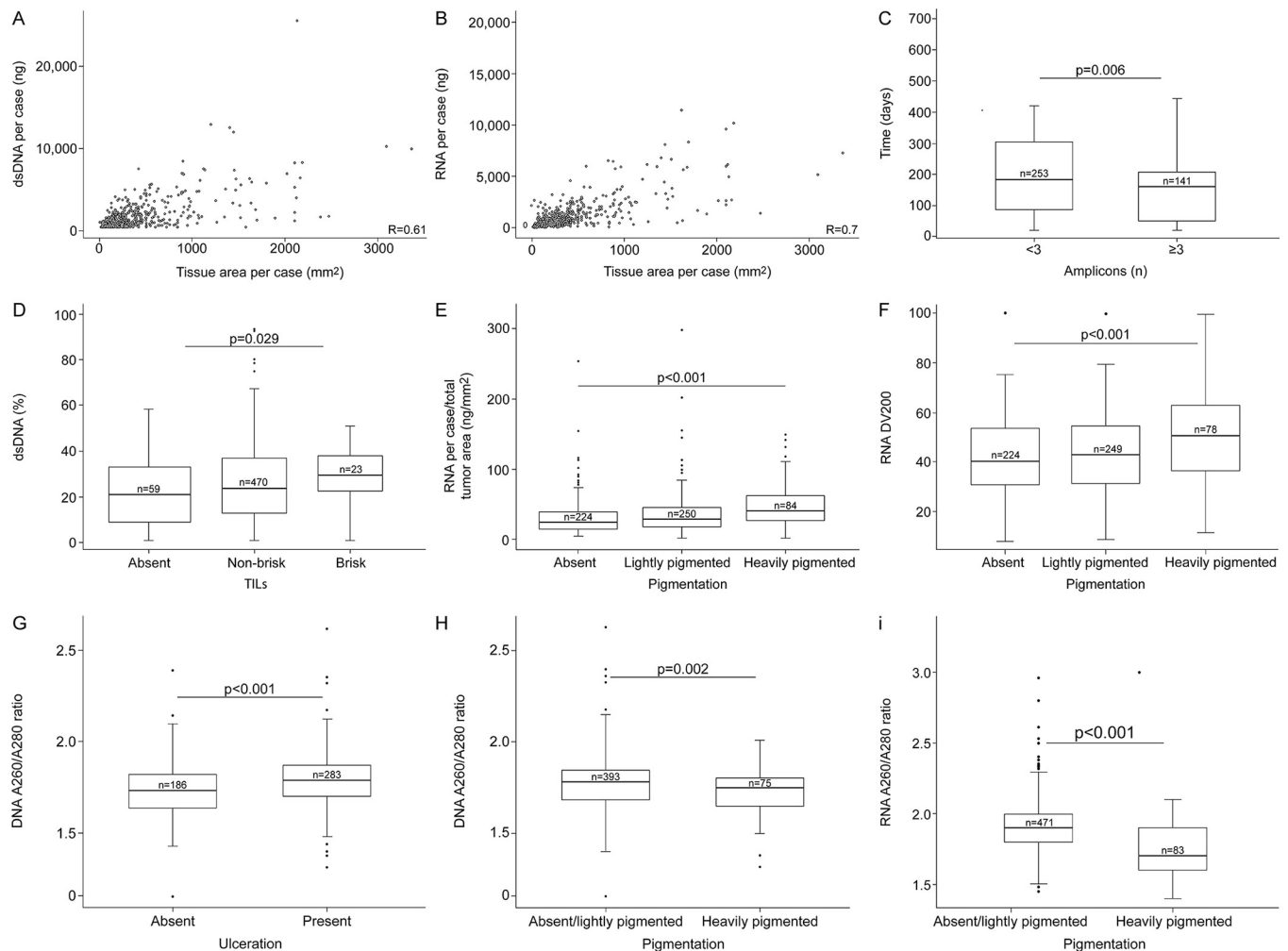
Fig 2. Effect of melanoma characteristics and time elapsed between tissue sectioning and co-extraction, on the quantity and quality of DNA/RNA.

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samples for methylation profiling with the Illumina Infinium MethylationEPIC arrays, and 560 (82%) DNA samples, for somatic mutation profiling by NGS with the MSK-IMPACT[TM] assay (Fig 4). In 446 (65%) cases, aliquots of RNA and DNA were distributed for testing with all three platforms. We compared the melanoma and tissue characteristics among cases with samples that qualified across testing platforms to evaluate potential biases or imbalances, and these characteristics are shown on Table 4.

Upon testing, 41 of 414 (10%) DNA samples with available methylation data for the present study failed QC due to low intensity probes (p>0.05) or insufficient single-sample Noob-normalization or BMIQ-normalization. We evaluated the effect of survival, marginally associated with failures (Fig 5A), to assess differences in the attrition between cases and controls. Characteristics associated with methylation screening failure were year of diagnosis (i.e. age of the blocks) (Fig 5B), time elapsed from sectioning to extraction (Fig 5C), %dsDNA (Fig 5D), and amplifiability (Fig 5D). Six of 683 (1%) RNA samples failed the Nanostring-based QC. These samples were flagged and then deemed unsuitable for data analysis because of their low proportion of probes above the minimal threshold. Inter-batch replicas showed a very similar miRNA expression profile before (r>0.8) and after correcting for batch effect (r>0.9). There were no variables found in association with Nanostring flags or failures.

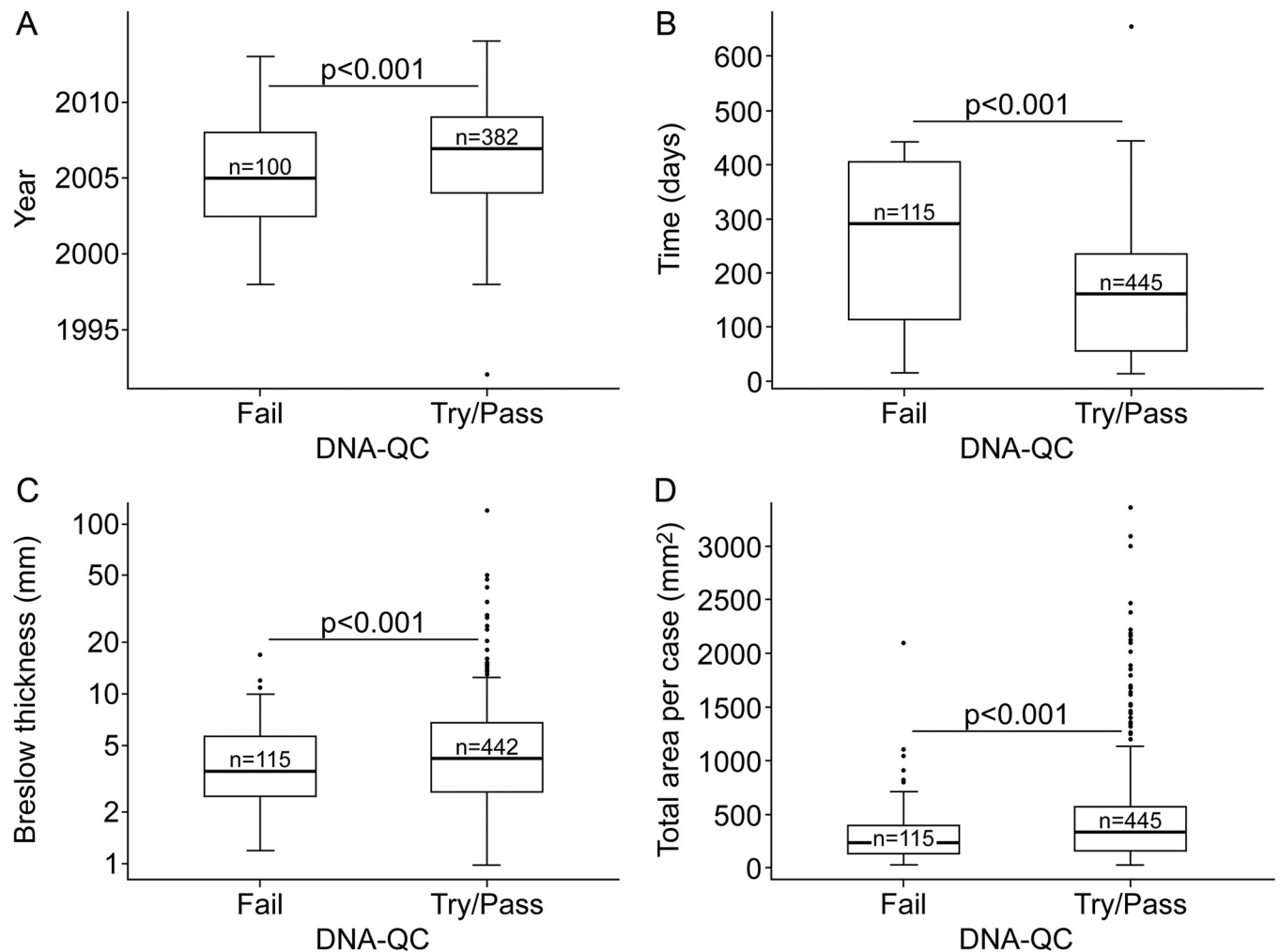**Fig 3. Effect of case and tumor characteristics, and time elapsed between sectioning and co-extraction on the core's DNA-QC score.**

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Of the samples sent for mutations screening with the MSK-IMPACT[TM] assay, 5 samples were subsequently deemed ineligible, 317/560 (57%) were run through the pipeline, and had data available for the present analysis. On average, the coverage was 249x, with a range of 22x to 762x, and 59 (18.6%) samples had coverage below 100x (Fig 4). The recommendations to proceed (or hold samples back) with the synthesis of libraries or MSK-IMPACT assay were significantly associated with coverage ($p < 0.01$) (S6 Fig in S3 Appendix); however, no significant associations were found between the amount of tissue extracted or any of the NA characteristics with samples scored as 'fail' by the genetics core (DNA-QC, Lib-QC). For details on data availability, see S1 Appendix.

Discussion

We have delineated a step-by-step approach for the handling of biospecimens and for data collection in a large multi-center international study, including quality assurance and quality control, in preparation for multi-omics testing from small sample size archival primary melanoma tissue. A unique aspect of this study is the testing of DNA and RNA co-extracted from the same cell lysate obtained from FFPE tissues utilizing conditions that are available in most
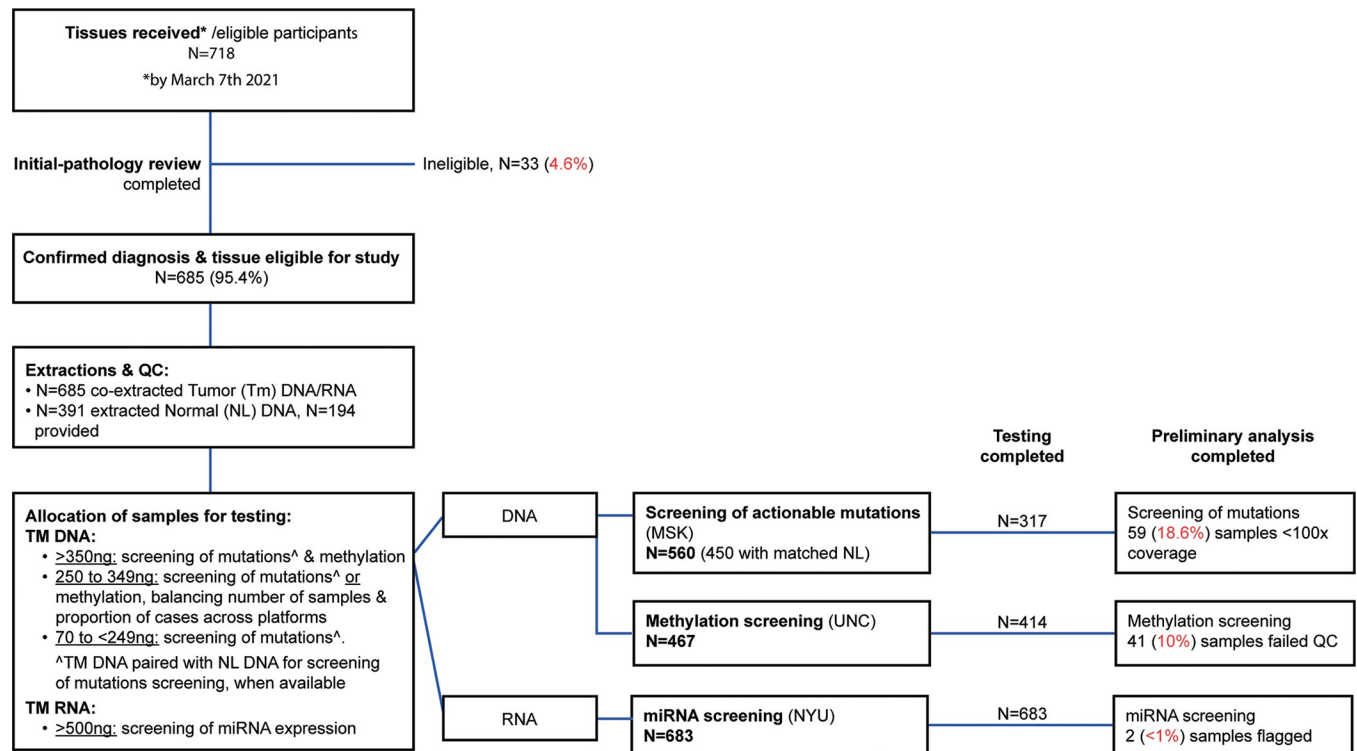
Fig 4. Biospecimens received, processed, and distributed for testing.

laboratories. This allows us to address the overarching goal of the Parent study, which is to integrate clinicopathologic, genetic and non-genetic data obtained across testing platforms. This co-extraction is critical for the proper integration of data obtained from multi-omics platforms utilized for the screening of mutations, methylation, and miRNA profiling, with protein expression and mRNA planned in the near future. We also provide an estimate of the anticipated attrition that will guide other large multicenter research and consortia.

FFPE tissue blocks of primary melanomas are extremely valuable as they represent the target lesion or 'index tumor' of interest and are accompanied by a vast array of clinicopathologic information. The process of formalin fixation and paraffin embedding helps conserve the cells topography, and blocks can be stored for decades without the need of ultra-low temperatures storage. However, it is well known that greater amounts and quality of nucleic acids may be obtained from fresh frozen tissues. Melanomas in particular, compared to other solid tumors, are most often very small at the time of diagnosis, and for purposes of accurate diagnosis the whole biopsy needs to be FFPE-processed. Thus, once diagnosis is completed as part of the standard care, only a limited amount of FFPE tissue remains available for research. While archived FFPE tissues are more likely to be available and more convenient for precision medicine, they present some challenges [18]. Because formalin forms cross-links during fixation that cause fragmentation during DNA extraction, and introduces contaminants, the use of FFPE can introduce artifacts in molecular profiling, although newer laboratory techniques, and analytical tools, help reduce the errors. It is generally accepted that nucleic acids obtained from old tissue blocks are suboptimal in quality and quantity; however, very few investigations considered the challenges in the context of co-extracted RNA and DNA for large studies using omics testing platforms, and none in small and/or pigmented tumors [19–22].

Table 4. Characteristics of participants and their melanomas for cases and controls with available full histopathology review and with samples allocated for testing, by miRNA, methylation, and mutation profiling platforms.

		All samples	Tested on all 3 platforms*	miRNA*	methylation*	NGS mutations*
N		561	396	559	414	482
Age						
	median years (IQR)	64 (52–75)	64 (54–75)	64 (52–75)	64 (53–75)	64 (53–76)
Sex						
	male	345 (61.5%)	248 (62.6%)	343 (61.4%)	262 (63.3%)	299 (62.0%)
	female	216 (38.5%)	148 (37.4%)	216 (38.6%)	152 (36.7%)	183 (38.0%)
Survival Group						
	survived ≥5 years	327 (58.3%)	212 (53.5%)	327 (58.5%)	225 (54.4%)	271 (56.2%)
	died <5 years	234 (41.7%)	184 (46.5%)	232 (41.5%)	189 (45.6%)	211 (43.8%)
Breslow thickness						
	median mm (IQR)	3.8 (2.5–6.2)	4.2 (2.8–6.8)	3.8 (2.5–6.2)	4.2 (2.7–6.8)	4.0 (2.5–6.5)
	not available	3	2	2	2	2
Ulceration						
	present	326 (58.2%)	248 (62.8%)	325 (58.2%)	258 62.5%)	289 (60.0%)
	absent	234 (41.8%)	147 (37.2%)	233 (41.8%)	155 (37.5%)	192 (40.0%)
	not available	1	1	1	1	1
Mitoses						
	present	522 (93.4%)	377 (95.2%)	522 (93.4%)	392 (94.7%)	453 (94.0%)
	absent	37 (6.6%)	19 (4.8%)	37 (6.6%)	22 (5.3%)	29 (6.0%)
	not available	2				
Pigmentation						
	absent/ not pigmented	224 (40.1%)	160 (40.9%)	223 (40.1%)	169 (41.0%)	193 (40.2%)
	lightly pigmented	250 (44.8%)	174 (43.9%)	249 (44.8%)	179 (43.5%)	214 (44.6%)
	heavily pigmented	84 (15.1%)	60 (15.2%)	84 (15.1%)	64 (15.5%)	73 (15.2%)
	not available	3	2	3	2	2
	absent/lightly pigmented	474 (85.0%)	334 (84.8%)	472 (84.5%)	348 (84.5%)	407 (85.8%)
	heavily pigmented	84 (15.0%)	60 (15.2%)	84 (15.1%)	64 (15.5%)	73 (15.2%)
	not available	3	2	3	2	2
Stage						
	II	293 (52.2%)	200 (50.5%)	292 (52.2%)	207 (50.0%)	252 (52.5%)
	III	268 (47.8%)	196 (49.5%)	267 (47.8%)	207 (50.0%)	230 (47.7%)

*N indicate availability of samples, data and data-analysis by the cut-off date. Allocation of samples for testing depends on the availability of tumor RNA (miRNA expression), tumor DNA (methylation), tumor and germline DNA (NGS, next generation sequencing for somatic mutations). For limited DNA, distribution for methylation or NGS contemplates survival group and overall balanced numbers across these two platforms. Proportions within each column sum up to 100% and do not consider the few unavailable datapoints, as shown above. Age, tumor Breslow thickness, and stage correspond to the initial diagnosis. Breslow and ulceration correspond to the maximum value reported either at diagnosis or during the centralized pathology review.

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Our priority is obtaining sufficient 'quality' DNA and RNA without exhausting FFPE blocks. In most cases, tumor tissues were procured as per our standard operating procedures, and when necessary fewer or greater number of sections were provided when necessary (due to scarcity of tissue or evidence of particularly small tumor areas). Non-tumor FFPE tissues, characterized by a lower cell (and nuclei) density, yield lower and less predictable amounts of DNA. Few centers have access to blood, buccal cells/saliva, or other tissue sources of non-tumor germline DNA. While surrounding tumor tissue may have some genetic change due to
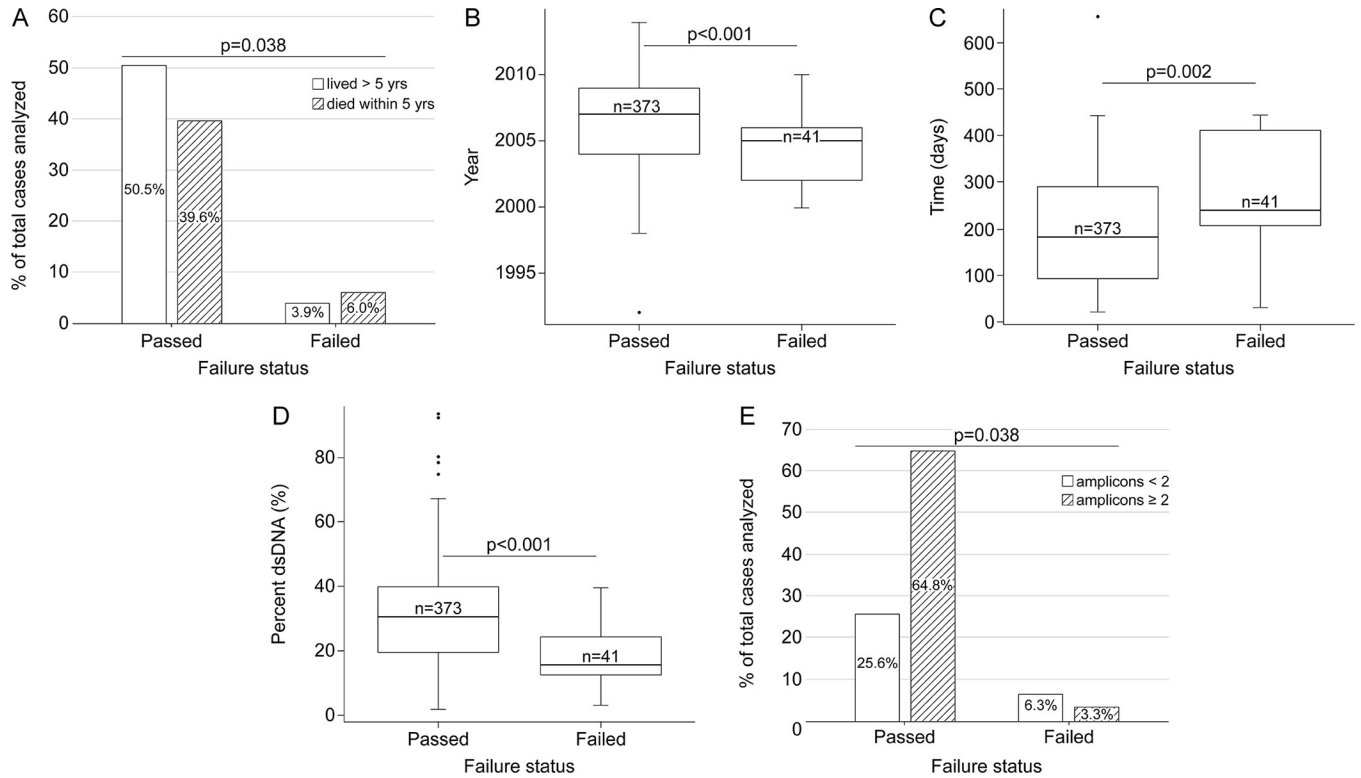
Fig 5. Effect of case, tumor, DNA characteristics, and logistics, on the success of methylation screening.

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field effects, it is still preferable to utilize adjacent normal tissue when no other source of germline DNA is available. We preferentially distribute tumor DNA paired with matched normal for somatic mutations profiling with MSK-IMPACT™; however, matched normal samples are not always available, or sufficient. While matched tumor-normal DNAs doubles the NGS costs, this approach provides 'clean' or accurate somatic mutation calling, free of germline artifacts. In addition, germline DNA allows the uncovering of potential mismatches, discrepancies, or contamination, through DNA fingerprinting. Of note, in this study we uncovered a very small fraction of discrepant samples, and even fewer remained unsolvable (0.9%) after rematching, or replacement of germline DNA. For melanomas in which the matching germline DNA is not available or the amount and/or quality inadequate, we submit tumor-only batches for analyses against a pool of normal DNA. To this effect, a robust pipeline has been developed to identify germline artifacts, taking into account coverage, allele level, copy number, and tumor purity (S4 Appendix). A related concern is the choice of volume for the elution of nucleic acids during the extraction (normal tissue) and co-extraction (tumors). On the one hand, greater eluates recover more DNA from the column but at lower concentrations, risking greater inaccuracy. On the other hand, with eluates that are too small, more DNA is lost to the column, capillary action, and to quantity and quality measurements. The ideal volume needs to be adapted according to preliminary co-extractions on similar tissues. Samples <20ng/ul are less reliable for determining purity accurately and this, plus much more stringent criteria, may contribute to the differences observed in the QC assessments obtained by our InterMEL Biocore for the NGS pipeline versus the CLIA-approved process that was developed by MSK for clinical use. Our observations and general success rate on NGS is in agreement with findings from others [23]. Although our average coverage is not exceptional (294x), preliminary

results from mutation screening in these co-extracted specimens demonstrates a similar profile to that found by TCGA [24].

In ideal conditions, tissues would render sufficient nucleic acids for QC and distribution to all three testing platforms. In our experience with small primary melanomas, 35% of the eligible cases had insufficient material for all 3 omics platforms: somatic mutations, methylation, and miRNA. Individually, 99.7% of the eligible cases had sufficient RNA for miRNA analysis, 78.8% for NGS, and 68.2% for methylation. To evaluate methylation, samples need to undergo an initial treatment with bisulfite, to convert unmethylated Cs to Ts. This treatment converts double-stranded DNA into more labile and unpaired single stranded DNA. Older tissue blocks and sections with more fragmented co-extracted DNA, will be naturally more susceptible to degradation, resulting in greater attrition, despite the use of a kit to restore degraded FFPE DNA (Fig 5). Melanomas with double-stranded DNA quantities below the threshold for methylation and mutation screening (~320 to 350ng double-stranded DNA), are adjudicated in nearly equal numbers for testing with one or the other DNA platform. In addition, in making this choice we considered their case-control status as well as availability (or not) of germline DNA for NGS. We also evaluated whether certain patient and tumor characteristics biased the distribution of samples across testing platforms. Age and sex were comparable across testing platforms, while some differences were noticed in the proportion of samples from patients who were stage III, died of melanoma within five-years, had thicker tumors, or with ulceration or mitoses (Table 4). The differences were not large, and these clinicopathologic variables will be included as covariates in downstream/formal statistical analyses.

Another important consideration is the timing of tissue sectioning. Because centralized block sectioning is not feasible, and most contributing centers are geographically distant from MSK, we wanted to minimize the time elapsed between sectioning and nucleic acid co-extraction, which is fully dependent on diagnostic confirmation/ histopathology review and marking of H&Es. The COVID-19 pandemic added unanticipated delays through complete closures in the first couple of months, followed by a few months of limited access to the research facilities, and major delays in the supply-chain, with sectioned tissues awaiting extractions for a very prolonged time. Indeed, the elapsed time had a detrimental effect on the DNA performance, which was evident through the ability to amplify fragments $\geq$300nt, synthesis of libraries for NGS, and the success of the methylation screening (Figs 2, 3 and 5).

Interestingly, we obtained greater amounts of RNA in heavily pigmented tissue. We posit that the melanin content decreases the pH, and that this may inhibit the enzymatic activity of the RNAses, otherwise ubiquitously present, an idea further supported by the greater proportion of RNA strands >200nt in heavily pigmented melanomas (Fig 2). Melanin is known to inhibit some enzymatic reactions [25,26] including amplification with Taq polymerase. Not surprisingly, samples obtained from heavily pigmented melanomas, on average, amplified shorter fragments (S4 Fig in S3 Appendix); however, we chose to not add an extra treatment to the samples as this would have affected the total yield. Unlike pigmentation, TILs and ulceration improved the DNA A260/A280 ratios, and samples from melanomas with brisk TILs showed greater %dsDNA and amplifiability, perhaps due to more actively dividing cells [27] resulting in increased cellularity. Samples with greater purity and %dsDNA were better scored by the core, but not those with higher RNA integrity. We have not found an explanation for this effect yet, although, no single variable or set of DNA variables was associated alone with the core's prediction for failure, or with the coverage obtained through the MSK-IMPACT™ assay. This differs to some extent to findings from others who reported block age as the most important variable that influenced sample viability for amplicon-based library construction [28]. For methylation, on the other hand, there are clear associations between year of diagnosis

(as a proxy of block's age), time elapsed between sectioning and extraction, %dsDNA, and amplifiability, with failures (Fig 5).

Another relevant aspect is the jurisdiction of the centers providing tissues and data. For instance, in 2020, a European center expressed interest in joining our efforts and contributing with a substantial number of cases. However, to comply with the General Data Protection Regulation 2016/679 (regulation in EU law, on data protection/privacy in the EU), a thorough analysis, numerous audits and reviews are taking place in relation to detailed information on (i) where specimens and data from EU will be located, (ii) who will have access to samples and data, (iii) how data and specimens are protected, and (iv) which systems and applications will be used to process, store, and run data derived for the samples from EU. This major delay may potentially affect the feasibility of including these specimens if samples cannot be procured and tested in time for analyses, especially since tissues cannot be sectioned in advance. International collaborations are important for ensuring worldwide representation of patient samples. Investigators should plan for these international regulatory steps, when planning future consortia.

In summary, we find only two variables that, to some extent, could be controlled in future studies of archival tumors and that have a detrimental effect on the quality of the extracted DNA from melanoma tumors: age of the FFPE tissue blocks, and time elapsed between block sectioning and co-extraction. We anticipated finding a set of tissue and derived nucleic acid features that would help predict testing success on multi-omics platforms, and thus spare the cost of samples carrying these features. Because of the large individual sample variability, we were not able to define any variable predictive of success on NGS; consequently, we will continue using a combination of quantity and quality parameters when choosing and preparing aliquots for screening DNA samples with the MSK-IMPACT[TM], and we will continue aiming to decrease the time elapsed between sectioning, extraction, and testing. The co-extracted RNA does not appear to be affected by the age of the block or time elapsed, but surprisingly, presence of melanin might impart some protection against RNA degradation in FFPE tissues. The greater RNA amounts and RIN values obtained in pigmented melanomas, to the best of our knowledge, are not artifactual, and are possibly due to a change in acidity or pH. Although pigment has no impact on the success of the miRNA screening with Nanostring, our observations are intriguing and worthy of further consideration. Of the 685 eligible cases, 65% of the melanomas rendered sufficient material for testing by all three omics platforms. The implication is that in future studies of early-stage melanomas using archival tissues, one should aim to accrue sufficient material to allow a 35% attrition, when the goal is to obtain genetic and epigenetic profiling such as ours. Furthermore, when considering the methylation failures, the ideal target would permit 40% attrition. We anticipate that these reported practical guidelines for the procurement, preparation, quality control, and testing of co-extracted DNA and RNA for the screening of actionable somatic mutations, methylation and miRNA using high-throughput platforms, as well as our data and insight will be of value to others with their ongoing and future investigations using valuable archival yet limited tissues.

Conclusions

Our experience on a large number of FFPE tumors demonstrates that, co-extracting nucleic acids from a common cell lysate obtained from FFPE tissues is a feasible approach for a large-scale study of genetic and epigenetic profiling. Caution must be applied during the early stages of the study design to account for 35–45% attrition of cases. We noticed that certain tissue features predict testing success differently for RNA and DNA; and that certain DNA features predict success for methylation but not for mutation screening; and presence of melanin affects

DNA but not RNA, although these findings need to be confirmed. With careful management of tissue processing and quality control, it is possible to conduct multi-omic studies in a complex multi-institutional setting for investigations involving small sized tumors, as in studies of very early-stage cancers.

Supporting information

S1 Table. InterMEL: Participants and melanoma variables and definitions.
(PDF)

S1 Appendix. Methods.
(DOCX)

S2 Appendix. Preliminary results.
(PDF)

S3 Appendix. Supporting figures. S1 Fig. Quantity and quality assessment of RNA and DNA co-extracted from FFPE. S2 Fig. Performance of RNA co-extraction kit and elution on Nanostring™. S3 Fig. Effect of FFPE tissue input on the yield of co-extracted nucleic acids. S4 Fig. Effect of TILs and pigmentation on DNA purity and amplifiability. S5 Fig. Effect of the DNA and RNA quality on recommendations to proceed with synthesis of libraries and NGS/MSK-IMPACT™. S6 Fig. Comparison of DNA- and library-QC recommendations with coverage obtained in melanomas screened with the MSK-IMPACT™ assay.
(PDF)

S4 Appendix. Letter of support.
(PDF)

Acknowledgments

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