Title:

Hope for Others: Research Results from the University of Pittsburgh Rapid Autopsy Program for Breast Cancer

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Abstract

Breast cancer affects 1/8 of women throughout their lifetimes, with over 90% of cancer deaths being caused by metastasis. However, metastasis poses unique challenges to research, as complex changes in the microenvironment in different metastatic sites and difficulty obtaining tissue for study hinder the ability to examine in depth the changes that occur during metastasis. Rapid autopsy programs thus fill a unique need in advancing metastasis research. Here, we describe our protocol and processes for establishing and improving the US-based Hope for OTHERS (Our Tissue Helping Enhance Research and Science) program for organ donation in metastatic breast cancer. As of August 2024, we consented 114 patients and performed 37 autopsies, from which we collected 551 unique metastatic frozen tumor samples, 1244 FFPE blocks, 90 longitudinal liquid biopsy samples and developed 14 patient-derived organoid and 8 patient-derived xenograft models. We report in-depth clinical and histopathological information and discuss extensive new research and novel findings in patient outcomes, metastatic phylogeny, and factors in successful living model development. Our results reveal key logistical and protocol improvements that are uniquely beneficial to certain programs based on identifiable features, such as working closely with patient advocates, methods to rescue RNA quality in cases where tissue quality may degrade due to time delays, as well as guidelines and future expansions of our program.

Statement of Significance

Rapid autopsy programs are unique research settings with huge potential for studying metastatic cancer, however, they have complex research challenges. Our work provides a valuable resource in advancing this field of research.

61 **Introduction**

62 Breast cancer affects 1 in 8 women throughout their lifetimes[1], with survival at five years
63 averaging 31% for patients who have distant metastases[2]. Despite significant gains in breast
64 cancer research and improvements in treatment in recent years, including the advent of CDK4/6
65 inhibitors and novel HER2-antibody drug conjugates, much work still remains to be done[3]. The
66 most lethal mechanism of breast cancer is metastasis, which is responsible for the majority of
67 cancer deaths, and addressing this challenge remains a critical focus of ongoing research
68 efforts[4,5].

69 However, metastasis is uniquely challenging to study, as it involves a complex interplay
70 between genetic and epigenetic modifications related to immune and other environmental
71 factors that are not easily captured in the laboratory setting. Clinical samples are urgently
72 needed as mouse models and other laboratory techniques may not fully capture the complexity
73 of human genetics and disease[6]. However, many clinical tissues that are biopsied are not
74 routinely preserved for research purposes and provide a limited number of organs and sites that
75 may not capture the full picture of metastasis[7,8].

76 Autopsies thus provide crucial diversity in the tissues collected for research purposes, which
77 leads to a more explicit understanding of the pathways taken during cancer metastasis[7].
78 Specific advantages include access to metastatic lesions that are challenging to biopsy, such as
79 bone; access from normal tissues to study organ tropism as well as intra/inter-organ
80 heterogeneity; larger amounts of tissue for in-depth molecular study/model development; and
81 the collection of tissue after lines of therapy to study drug resistance – a major challenge in
82 breast cancer treatment[9].

83 The establishment of a tissue donation program at UPMC Magee Women's Hospital was driven
84 by patient requests within the Breast Cancer Program, a component of the NCI-designated
85 UPMC Hillman Cancer Center and the Magee Women's Hospital. Between 2008 and 2015, four
86 patients with metastatic breast cancer nearing the end of their lives expressed a desire to
87 contribute to scientific research through body donation[10]. While these initial requests were
88 accommodated, the process lacked structure and organization. The development of this
89 structured tissue donation program was motivated by the need to streamline the process,
90 maximize the scientific value of donated tissues, and fulfill the wishes of patients who sought to
91 contribute to the advancement of breast cancer research even after their passing.

The significant quantity and quality of tissue obtained from these autopsies, coupled with the recognition that serial tissue collection throughout metastatic breast cancer progression enhances the value of autopsy tissue, highlighted the need for a formalized and proactive tissue procurement program. This initiative aimed to gather tissue samples throughout the illness and to ensure efficient and timely tissue collection at death, thereby honoring the patients' desire to leave a lasting impact on cancer research and, thus, on future patients suffering from the disease through improved understanding of breast cancer evolution, heterogeneity, and metastases.

At the program's inception, no cancer-specific tissue autopsy procurement programs existed within our academic center. However, an existing rapid autopsy program for Idiopathic Pulmonary Fibrosis (IPF) patients served as a valuable model[10]. This IPF program helped identify crucial departments, personnel, and procedural workflows necessary for conducting autopsies effectively[11], and other major logistical changes that need to be implemented. Hence, a major re-design was implemented in 2018, leading not only to exponential increases in consents and autopsies but also to increases in the quality and quantity of tissue collection and research progress.

This study presents a detailed analysis of the development, implementation, and outcomes of our rapid autopsy program, addressing logistical challenges and highlighting solutions. We report on diverse causes of death in metastatic breast cancer and emphasize the importance of systematic tissue collection, including the discovery of micro-metastases in grossly normal organs. Methodological advances in tissue preservation, particularly fixed sequencing technologies for RNA integrity in post-mortem samples, are discussed alongside the development of patient-derived organoids (PDOs) and xenografts (PDXs). Novel findings, such as the identification of an ESR1-ARNT2 fusion in metastatic samples from one patient, demonstrate the program’s potential to uncover new molecular features of metastatic breast cancer. Our experience provides valuable insights for improving rapid autopsy protocols and advancing metastatic cancer research globally.[12]

Results

Overall logistics of the HfO program from consenting to tissue processing

Our process for the Hope for OTHERS (Our Tissue Helping Enhance Research and Science) Tissue Donation Program illustrated in Figure 1 begins with the patient learning about and consenting to the program, which can occur years to days before their passing. Previous research from our group has shown that many patients are willing to discuss autopsy, but care providers must initiate such conversations[13]. As of August 2024, our average time from consent to death is 14-15 months, with a range of 0 to 53 months and a median of 10 months (Figure S1). Upon consent, we integrate the collection of longitudinal samples such as blood, ascites, and biopsies throughout the patient's treatment journey flagging them in coordination with the clinical team and biobanking them where possible. These samples are logged and preserved via the Pitt Biospecimen Core, allowing for comprehensive temporal analysis of cancer progression.

As the patient's condition declines, pathology and lab teams are alerted and prepared for potential tissue collection. Upon the patient's death, the program enters a critical 3-7 hour window. The research coordinator is promptly informed and contacts a livery service to transport the body to the morgue. Simultaneously, pathology, radiology, and lab teams are notified about the impending autopsy. Once the body arrives at the morgue, we collect a post-mortem CT (computed tomography) scan.

The final phase, lasting 1.5-3 hours, involves the actual tissue collection. The autopsy begins, and the lab team works to preserve specimens using various methods. These include snap freezing, FFPE (Formalin-Fixed Paraffin-Embedded) preservation, cryopreservation, and the initiation of PDX (Patient-Derived Xenograft) and organoid development. Following collection, tumor samples are transported in batches to the lab for further processing.

As of August 2024, our program has consented 114 patients and completed 37 autopsies, averaging 5.5 autopsies per year since 2018. Our patients' clinical characteristics (Table 1) reflect the general population demographics with ER+, PR+, HER2- being the most common molecular subtype. NST (no special type) is more prevalent than ILC (invasive lobular carcinoma) which also roughly approximates the frequency of ILC vs NST in the general population (~15%) at 4/30 (~13%) patients (Table 1). Stages at diagnosis ranged from 1A to 4. Our patients are most frequently diagnosed in stage 2A.

150 Figure 2 presents a comprehensive timeline of treatments and outcomes for the 30 patients
151 from the post-2018 autopsies [pre-2018 not included due to inconsistent data prior to the 2018
152 program re-design], each represented by a horizontal bar plotting their treatment and
153 progression information, normalized by total duration from the patient's initial diagnosis to the
154 time of death. The first five squares color-code the characteristics of the patient for primary
155 tumor molecular subtype, race, gender, histological subtype, and stage at the time of diagnosis.

156 Lastly, the treatment timeline uses color-coded bars to represent different therapies, including
157 hormonal therapy, chemotherapy, targeted therapies, immunotherapy, and various inhibitors
158 and conjugates. Simultaneous therapies are defined by vertically stacking bars. Our data shows
159 that most of our patients, with some exceptions, follow similar treatment lines for their disease
160 subtype. Symbols on the bars represent treatment markers, with an increasing density of red
161 triangles later in treatment reflecting an increase in the progression rates in late-stage disease.

162 **Patient advocates play a crucial role in improving perceptions of the program**

163 The development of rapid autopsy and organ donation programs for breast cancer research is a
164 sensitive undertaking that requires careful consideration of ethical, emotional, and practical
165 concerns. As we embarked on this initiative, it quickly became apparent that the perspectives of
166 those most intimately affected by breast cancer—the patients themselves—were indispensable.
167 Recognizing the delicate nature of discussions surrounding end-of-life care and post-mortem
168 tissue donation, we realized that incorporating breast cancer advocates into our program was
169 not just beneficial, but essential. These advocates, often breast cancer survivors and/or
170 individuals with close ties to the breast cancer community, bring a unique and vital viewpoint to
171 the table. Their involvement ensures that our approach remains patient-centered, addressing
172 the concerns and honoring the wishes of those who might consider participating in such
173 programs.

174 Hence, as an extension of our process, our group specifically incorporated a group of patient
175 advocates with metastatic disease to represent patient voices on the leadership committee. This
176 has led to several notable improvements, as discussed below.

177 This group coordinated a rebranding of our program to The Hope for OTHERS (Our Tissue
178 Helping Enhance Research & Science; HfO) Tissue Donation Program. The new name was
179 carefully chosen to reflect the altruistic nature of tissue donation and its critical role in advancing
180 scientific understanding of breast cancer.

181 As part of this rebranding effort, we developed an independent, patient-focused website and
182 created new materials such as brochures and pamphlets (Figure 3a). These resources were
183 designed to provide clear, compassionate information about the program to potential
184 participants and their families.

185 Our patient advocates have become integral members of the team, participating in our larger
186 Hope for OTHERS meetings and holding additional meetings among themselves to discuss
187 program improvements and outreach strategies. Their involvement ensures that patient
188 perspectives are consistently represented in all aspects of the program. For example, we have
189 recently participated in a podcast raising awareness of our program, which has been
190 downloaded 330 times to date across 17 countries as of August 2024 (Figure 3a).

191 To increase awareness and engage with the broader breast cancer community, our program's
192 patient advocates have been actively presenting and distributing our materials (Figure 3a) at
193 various regional and national conferences, such as the Metastatic Breast Cancer Research
194 Conference 2024, Living Beyond Breast Cancer 2024, as well as advocacy events such as the
195 2024 Komen Pittsburgh More Than Pink Walk (Figure 3b).

196 Working closely with the advocate team and collaborating with groups across the country, we
197 have seen a significant increase in interest in tissue donation for research purposes in
198 metastatic breast cancer. Improving the perception of the program and its goals through
199 frequent bidirectional interactions ensures that the patient perspective remains central to our
200 efforts, furthering our mission of advancing breast cancer research through the normalization of
201 patient donations.

202 **A clinical coordinator specifically dedicated to the HfO program assures communication**
203 **and increases program efficacy**

204 Rapid autopsy programs require engagement with numerous stakeholders, including but not
205 limited to multiple clinical and basic departments, patient advocates, patients' families, industry
206 collaborators, researchers, regulatory offices, and funding agencies. The multi-layered
207 complexities of the program require the commitment of and oversight by scientists who are truly
208 vested in the success of the program, for example, those with a research focus on metastatic
209 breast cancer, as is the case in our program.

210 An essential improvement of our program has been the addition of a dedicated clinical
211 coordinator. The clinical coordinator is an integral part of the program, interacting with care

212 providers, patients, and their families, pathologists on call, livery service, and the lab specimen
213 processing team (Figure 1). While factors such as travel time for patient transport are outside of
214 the coordinators control, we are only able to keep a narrow and consistent autopsy (Mean:
215 132.15 minutes, Standard Deviation: 49.23 minutes) and processing time (Mean: 137.03
216 minutes, Standard Deviation: 63.44 minutes) due to coordinator communication between the
217 research lab and the autopsy team (Figure 3c), as well as maintain a steady increase in rates of
218 consents due to their integrated and dedicated role in the clinic (Figure 3d).

219 We have established a structured meeting schedule to maintain program flexibility and
220 continuous improvement. Biweekly meetings with the active operational group focus on case
221 discussions, areas for improvement, and research progress monitoring. Bimonthly meetings
222 involving all multidisciplinary team members and major stakeholders allow for sharing results
223 and discussing larger-scale improvements, such as annual reviews of our standard operating
224 protocols. A major re-design of roles and responsibilities, in 2023, by shifting more research
225 responsibilities to dedicated research and autopsy coordinators, led to a dramatic increase in
226 our rate of consents by the clinical coordinator due to a more focused and narrow scope as a
227 result (P-value 6.38E-31) (Figure 3d).

228 **Comprehensive and Diverse Tissue Collection Enhances Longitudinal Metastatic Breast**
229 **Cancer Research via Rapid Autopsy**

230 At autopsy, we currently prioritize three collection modalities:

231 1. FFPE cassettes
232 2. Snap-frozen tissue for molecular analyses
233 3. Cryopreserved tissue for the development of patient-derived organoids (PDOs) and
234 patient-derived xenografts (PDX)

235 As of August 2024, we have collected a sum total of 1244 FFPE blocks, with a median of 41 per
236 patient (range of 7 – 69) (Figure 4a).

237 We have also collected 511 unique frozen tumors, with a median of 12 per patient (range of 3-
238 27) (Figure 4b). A key strength of our program is the ability to access the original primary tumor
239 samples for 25 of our cases (64%), despite sometimes being decades between primary surgery
240 and death. This, combined with intermediate samples such as liver biopsies from the clinic,
241 creates unique opportunities for longitudinal studies examining the evolution of metastatic

242 breast cancer. Lastly, we have also collected 1952 cryovials, with a median of 46 per patient,
243 and a range of 0 to 142 (Figure 4c).

244 Our research strategy also prioritizes the longitudinal capture of patient data, which can then be
245 linked to future research studies; this includes both clinical data and, more importantly, liquid
246 biopsy blood collections at each progression. These collections are a result of close
247 coordination and collaboration with the medical oncology department and allow us to keep track
248 of patient status, treatment progression, and other important clinical notes that might otherwise
249 be missed and consolidate them for consistent formatting inclusive of the clinical context of each
250 progression. For our 37 cases, we have collected 90 total, with a median of 2 per patient and a
251 range of 1 to 8 progression blood collections (Figure 4d).

252 A primary improvement due to our autopsy process has been a noted increase in the diversity of
253 tissue collected compared to previous studies in metastatic tissue. Even in studies that
254 prospectively select for metastatic tissue, such as the 91 patients with metastatic breast cancer
255 in the MET500 study, biopsies for liver and lymph nodes are overwhelmingly the majority of
256 samples collected (61.9%)[14] due to ease of access in the clinical setting (Figure 4e). Our results
257 highlight the advantage of rapid autopsy programs to increase diversity of tissue samples
258 collected.

259 As of August 2024, our program has collected from 228 organ sites, with fairly equal
260 representation from many tissue sites, with liver (12.7%), lung (10.5%), and spleen (8.8%),
261 making the top three showing relatively similar levels of collection. Furthermore, we also show
262 increased diversity with rare sites of micro-metastases or local invasion such as thyroid,
263 bladder, and diaphragm also being collected. We have collected from 29 total unique tissues
264 compared to 11 (AURORA US Clinical Samples), 18 (MET500), and 11 (AURORA EU) from
265 programs that collect using clinical biopsies (Figure 4e)[7,14,15]. This emphasis on comprehensive
266 sampling via an autopsy approach has allowed us to capture a more complete picture of
267 metastatic progression and organ involvement. All tissues are kept in -80 or -150 freezers and in
268 duplicate both in our lab and at the Pitt Biospecimen Core to ensure backups in case of power
269 outage or other system failures.

270 **Differential causes of death in breast cancer necessitate consistent and diverse tissue**
271 **collection.**

272 Recent research has identified that causes of death in metastatic breast cancer are varied and
273 require further examination[16]. To investigate this, we conducted a systematic review of our

274 clinical records and autopsy reports after noting discrepancies between patient symptoms and
275 gross clinically detected metastases. We focused specifically on lab values and medical notes
276 from the last six months of life as well as the final autopsy report after death.

277 This analysis showed that the primary cause of death in the majority of patients enrolled in the
278 HfO program was due to liver or respiratory dysfunction secondary to breast cancer metastasis
279 (Figure 5a). However, while the majority (90%) of the liver pathologies were similar, with
280 consistent signs of hyperbilirubinemia, portal hypertension, and cardiac strain, lung pathologies
281 were much more diverse in the proximal cause, including but not limited to chronic kidney
282 disease secondary to pulmonary congestion, disseminated intravascular coagulation, saddle
283 pulmonary embolism, pneumonia, enhertu-related pneumonitis, and pleural effusions. Some,
284 such as saddle pulmonary embolisms, have known or hypothesized causes currently under
285 study[17]. Critically, many organ systems were revealed to have detrimental effects on others,
286 such as the liver on the kidney due to breakdown of blood supply, or how pleural effusions or
287 hepatojugular reflux could each cause cardiac strain. This illustrates how disentangling the
288 specific cause of death in multisystem organ failure due to complications associated with
289 metastatic breast cancer is a challenging task. Our data shows that causes of death in patients
290 can vary in pathology and urgently demand further precise investigations into the underlying
291 biology.

292 **Microscopic tumor metastasis is more common than expected and has different patterns**
293 **between breast cancer histological subtypes.**

294 Often, the differences in metastatic organs can be microscopic. A review of all grossly normal
295 organs from our patients showed that, on average, at least one additional organ from a patient
296 may have micro-metastases not visible on clinical imaging (Figure 5b).

297 These have important implications for the journey of metastases and systemic responses to
298 these metastases in different immune tissues. In some cases of ILC, patients were deemed to
299 have ‘normal liver’ on CT repeatedly up until death, necessitating orthogonal approaches such
300 as identification of lesions using ultrasound and MRI (magnetic resonance imaging); (Figure 5c,
301 5d) only to reveal at autopsy multiple metastatic lesions affecting the hepatic parenchyma
302 despite consistently ‘normal’ CTs (Figure 5e, 5f) – a finding consistent with current ILC research
303 and emphasizing the need for improved imaging as well as orthogonal methods of metastatic
304 validation in addition to imaging[18].

305 This kind of discrepancy was observed to be common in our patients with ILC, where numerous
306 peritoneal organs often have metastases that are not visible clinically, as seen in Figure 5g. As
307 a result, we made changes in our collection protocol (see Supplemental 1) that have resulted in
308 increased standardized collection of lung, liver, brain, and bone in patients with NST and
309 peritoneal organs and tissues in patients with ILC, with clear anatomical labels and records,
310 increasing data representation overall for these diseases (Figure 5h). In addition, the need and
311 desire to understand dormancy, especially in patients with late recurrences, such as in the case
312 of ILC[19], has prompted us to increase the collection of macroscopically normal tissue, such as
313 bone, lung, and spleen, as well as sites considered to be 'commonly' involved in cancer
314 metastasis – even if grossly normal (Figure 5i).

315 **Fixed single cell sequencing technologies are preferred for tissues with degraded RNA in**
316 **the autopsy context**

317 Previous research from Geukens et al. has shown that bulk RNA quality in tumor tissue
318 decreases rapidly within hours of time of death[20]. In order to explore this and extend this work,
319 we performed paired single nuclei sequencing from three liver samples using 3' chemistry from
320 frozen tissue that was snap frozen at autopsy, and using fixed flex technology from 10x
321 Genomics on matched fixed tissues that were fixed and paraffin embedded at autopsy. Fixed
322 flex uses multiple probes to identify fragments and multi-align probe signatures[21].

323 Our results showed significant degradation of RNA from frozen tissues, which is in line with
324 findings from previous research that RNA quality degrades in an autopsy setting[20]. At the same
325 targeting of 8000 cells, on average, 7920 cells (SD: 1115) pass quality control for fixed
326 sequencing, but only 6959 (SD: 585) pass quality control for frozen sequencing.

327 Additionally, fixed sequencing technologies can rescue some of the signals, resulting in an
328 increased number of genes detected. Fixed sequencing detects an average of 2408 genes
329 [95% CI 2387 - 2430] vs. 1454.77 [95% CI 1440.59 – 1468.95] in frozen sequencing. Fixed
330 sequencing also resulted in increased total molecular counts with an average of 3158.05 [95%
331 CI 3145.28 – 3170.81] total counts vs an average of 2357.83 [95% CI 2347.03 – 2368.63].

332 Lastly, we also see decreased mitochondrial contamination with an average of 0.65% mtDNA
333 percentage [95% CI 0.64 – 0.65] vs 0.92% [95%CI 0.91 – 0.94] (Figure 6a).

334 Here, we see that fixed technologies also reduced noise, as these samples integrate better in
335 the case of fixed technologies. They also show a wider variety of cellular populations with more

336 significantly increased heterogeneity (Figure 6b and Figure 6c) – crucially Figure 6b reveals that
337 even among samples processed together, degradation from the autopsy itself contributes to
338 significant divergence in UMAP clustering and that in frozen technologies these differences are
339 too large to be disentangled using Harmony, but are correctable in fixed sequencing in Figure
340 6c. We also see an increase in signal detection, as ESR1, and CD4 for example are higher
341 detected (Figure 6c) when using fixed sequencing.

342 Downstream analysis is also impacted, in Figure 6d we integrate the samples and label by cell
343 type. For quality assessment, we exclude the cancer cells specifically, as inherent cancer
344 subclone heterogeneity and the expression of neuronal and stem-like markers common in
345 cancer cells are confounding factors in this analysis comparing sequencing techniques, as
346 single-cell sequencing analysis is unable to reliably deconvolute the number of subclones
347 present and how much of the variation is due to inherent cancer heterogeneity vs. technique
348 differences. We do not have such significant variations in the ground truth in other cell types
349 which allows us to more accurately assess the quality of each sequencing technique. We then
350 run side by side pathway analysis on cell type populations identified in both groups and show
351 significant reduction in pathway activity detection in the Hallmark pathways.

352 While the same pathways are present in both fixed and frozen data in Figure S2, there is always
353 a decreased gene set percentage in frozen data and a decreased pathway score in all four cell
354 types, indicating worse signal quality (Figure S2).

355 **Time from death of patient to processing tissue is critical for developing living models**
356 **that have the potential to reveals metastatic disease evolution.**

357 Through our HfO program, we have established additional corollaries for PDO and PDX
358 generation. Specifically, we have been successful in generating 14 PDOs from 7 patients, and 8
359 PDX from 4 patients, covering a range of molecular and histological subtypes.

360 Across 27 attempts with 13 successes at developing PDOs, logistic regression revealed time to
361 end of processing was a significant factor in organoid growth success, with the latest success in
362 our tests being at 9 hours (Figure 7a) and a coefficient of -0.0132 for each additional minute of
363 delay from time of death to end of processing ($p = 0.027$). We have also recently had significant
364 success with 4 cryopreserved PDO developments out of 5 attempts and are currently
365 accelerating new attempts from prior banked samples to unlock the potential of past collections
366 further. To increase the possibility of success of organoid development, in 2023, our updated
367 SOP (Supplemental 1) changed to submerge all organs whole in pre-chilled DMEM, drawing

368 inspiration from organ transplant protocols[22]. Work to quantify the improvement as a result of
369 this change is ongoing.

370 We are also collaborating with Champions Oncology on the generation of PDX models, with 11
371 patients, 34 attempts, and 8 successes, with no successes past the 10-hour mark after the time
372 of death (Figure 7c). Lastly, logistic regression confirmed a commonly known factor in PDX
373 success in triple negative vs. ER+ tumors, with coefficient of -2.8194 and p-value of 0.017,
374 confirming prior findings that tumors that are ER+ have greater difficulty in engrafting[23] (Figure
375 7b).

376 Furthermore, our collaboration with Champions has led to the generation of a custom protocol
377 for excising tissue chunks for PDX implantation – noting that minced tissue is preferred for PDO
378 generation, but chunked whole tissue is preferred for PDX generation (Figure S3).

379 Hence, careful evaluation of the number of each type of tissue preservation is essential to
380 maximize downstream research options while operating with time constraints to transfer tissues
381 in media and/or on ice as fast as possible.

382 **Novel research findings and the importance of collaboration with external partners.**

383 These PDX models have also given us unique opportunities for the study of metastatic
384 progression. For example, we made the novel discovery of an *ESR1-ARNT2* fusion in a PDX
385 model (Figure 8a) generated from a sample from a patient who participated in the HfO program.
386 We then found the *ESR1-ARNT2* gene fusion to be ubiquitous among the metastatic tissues
387 collected from our program for that patient (Figure 8b). We were subsequently able to cultivate a
388 patient-derived xenograft organoid (PDXO) from this model (Figure 8c), and demonstrated that
389 both the PDX and PDXO expressed the *ESR1-ARNT2* fusion gene (Figure 8d). We were able
390 to express this fusion in cell-lines (Figure 8d), and are now studying it in greater detail. This then
391 helps us build on previously published work from our lab as well that of other labs demonstrating
392 that ESR1 fusions have unique activity and frequency in metastatic ER+ breast cancer tissue[24–
393 26].

394 The acceleration of our consent and autopsy progress has also been reflected in our research
395 efforts. As of August 2024, we now have multiple projects in progress, including but not limited
396 to research efforts looking at dormancy, genomic structural evolution, leptomeningeal
397 metastasis, expansion of PDX model development to look at specific drug resistance models,
398 ESR1 mutant effects in the liver microenvironment, and international collaborations at the

clinical and molecular history of changes in breast cancer subtypes such as ILC with the UPTIDER program[20]. We expect critical discoveries in the coming years from the growing number of autopsy programs including ours. It's important to note that the nature of these programs is highly dependent on the rate of sample collection and quality of data collected for research productivity and thus requires a certain degree of establishment before research output can catch up, which we have only recently managed to reach. This kind of investment requirement has significant funding and research planning implications**.**

Discussion

The Hope for OTHERS program represents a breakthrough in metastatic breast cancer research. Our findings highlight the importance of rapid autopsy programs for understanding metastatic progression, while revealing challenges requiring multidisciplinary collaboration. Many programs fail without securing necessary support from the start, explaining why initiatives like ours remain rare globally[12].

Programmatic Improvements and Their Impact

A dedicated clinical coordinator has been crucial to our success by facilitating stakeholder communication and streamlining processes, resulting in increased patient consent and completed autopsies. The participation growth (Figure 3d) demonstrates this approach's effectiveness as a potential model for other institutions.

Collaboration with patient advocates transformed our program through improved branding, better communication materials, and increased public awareness. This patient-centric approach has boosted participation while ensuring our research aligns with patient needs.

Challenges and Solutions in Program Logistics

Transportation time challenges (Figure 3c) emphasize the need for flexible protocols in rapid autopsy programs. Our experience shows population density significantly impacts efficiency. Institutions with similar geographical challenges may benefit from adapting their protocols accordingly.

Our interdepartmental collaborations, particularly with Pathology and Radiology, have been essential in maintaining short time-to-autopsy windows and enhancing data collection quality, demonstrating the importance of an institution-wide approach.

Scientific Insights and Methodological Advances

429 Our findings regarding the diverse causes of death in metastatic breast cancer (Figure 5a)
430 underscore the complexity of the disease and the need for comprehensive tissue collection
431 protocols. The discovery of micro-metastases in grossly normal organs (Figure 5b) highlights
432 the importance of systematic sampling, even in apparently unaffected tissues. This approach
433 has particular relevance for specific subtypes like ILC, where peritoneal (and other) metastases
434 may be clinically occult (Figure 5g, 5h) and dormancy is a very critical yet unresolved issue.

435 Other improvements include rapid cooling of organs in medium to slow metabolic activity and
436 molecular degradation[20]; consistent collection of all major organs even if grossly normal (Figure
437 5h); prioritization of tissues of key research interest, such as the leptomeninges.

438 The development of subtype-specific collection protocols (Figure 5h) represents a significant
439 methodological advance. This tailored approach ensures more consistent and relevant tissue
440 collection, potentially leading to more robust and representative datasets for future studies.

441 Our exploration of RNA quality preservation techniques (Figure 6a-d) provides valuable insights
442 for researchers facing similar challenges with post-mortem tissue quality. The superior
443 performance of fixed sequencing technologies in preserving RNA integrity and cellular
444 heterogeneity information could inform future methodological choices in single-cell studies using
445 autopsy tissues. Fixed sequencing technologies clearly decrease signal loss, as signal
446 integration and removal of batch effects are much clearer in fixed analysis. We suspect that part
447 of the batch effect is from the continued RNA degradation during extraction and lysis of cells to
448 isolate single nuclei, while fixed RNA is more stable, and any FFPE artifacts are rescued by the
449 redundancies in probe signals. In situations where tissue quality may be subpar or RNA quality
450 is expected to be degraded due to autopsy-related factors, fixed sequencing technologies are
451 preferred in single-cell applications. Fixed sequencing technologies, however, are not perfect,
452 as we do note that certain signals, such as CD8, are not detectable in either set of analyses,
453 and there is still significant RNA degradation compared to fresh single-cell sequencing collected
454 in optimal conditions such as surgical resections[27].

455 The time-sensitive nature of PDO model generation from autopsy tissues (Figure 7a) offers
456 crucial guidance for researchers aiming to develop living models from rapid autopsy programs.
457 These findings can help optimize tissue processing protocols and set realistic expectations for
458 model development success rates. Important to note is the high variability in PDX success even
459 within the same patient, implying significant factors other than time in PDX development and
460 ER+ status that need to be further optimized. Work to establish the effect of tissue type is

currently ongoing. The range of successes within patients also indicates that tissue quality based on blood supply, technique and other similar factors could significantly impact the success of PDX generation (Figure 7c).

Novel Findings and Future Directions

The identification of the ESR1-ARNT2 fusion (Figure 8b) exemplifies the potential of rapid autopsy programs to uncover novel molecular features of metastatic breast cancer. This finding, along with our ongoing studies on ESR1 fusion functions, demonstrates how rapid autopsy programs can drive forward our understanding of treatment resistance and metastatic progression. Both models are valuable and available to collaborators for further research, emphasizing the importance of models developed from programs like ours.

The diverse range of ongoing projects stemming from our program, including studies on dormancy, leptomeningeal metastasis, and rare subtypes like ILC, showcases the broad impact of comprehensive rapid autopsy programs on breast cancer research and the potential for new discoveries previously unknown.

Limitations and Future Considerations

Despite our successes, we acknowledge several limitations. The single-institution nature of our study may limit the generalizability of some findings. Specifically, inter-institutional variations in protocol may also lead to differences in downstream research results[12]. Additionally, while we've made strides in reducing time-to-autopsy, further improvements could enhance tissue quality and model generation success rates.

Future directions for our program include expanding collaborations with other institutions to increase sample diversity and validate our findings across different populations. We also aim to refine our tissue collection and processing protocols further based on emerging technologies and research priorities.

In conclusion, the Hope for OTHERS Tissue Donation Program demonstrates the profound impact that well-designed rapid autopsy programs can have on advancing metastatic breast cancer research. By sharing our experiences, challenges, and solutions, we hope to contribute to the broader effort of improving rapid autopsy protocols, increase patient enrollment and ultimately advancing our understanding of metastatic breast cancer biology.

Clinical Characteristics		
Primary Molecular Subtype	ER+/HER2-	22 (59.4%)
	ER+/HER2+	6 (16.2%)
	ER-/HER2+	1 (2.7%)
	TNBC	7 (18.9%)
Race	White	35 (94.6%)
	Black	2 (5.4%)
Gender	Female	36 (97.3%)
	Male	1 (2.7%)
Histological Subtype	NST	33 (89.2%)
	ILC	4 (10.8%)
Stage at Time of Diagnosis	I - II	19 (51.4%)
	III	9 (24.3%)
	IV	4 (10.8%)
	Unknown	5 (13.5%)

Table 1: Patient and Tumor characteristics of the HfO cohort as of August 2024

495 **Methods**

496 All dates and statistics are as of a freeze date of August 1st, 2024.

497 **Operating Protocol**

498 Please see attached Supplemental 1.

499 **Frozen single nuclei extraction**

500 *Reagents and Buffers*

501 Nuclei isolation was performed using the following reagents: Trizma® Hydrochloride Solution
502 (1M, pH 7.4; Sigma T2194), Sodium Chloride Solution (5M; Sigma 59222C), Magnesium
503 Chloride Solution (1M; Sigma M1028), Nonidet™ P40 Substitute (Sigma 74385), Phosphate-
504 Buffered Saline (PBS) with 10% Bovine Albumin (Sigma SRE0036), and Protector RNase
505 Inhibitor (Sigma 3335399001).

506 Two buffers were prepared:

507 Lysis Buffer (TST): Composed of 1X ST buffer (10 mM Tris-HCl, 146 mM NaCl, 21 mM MgCl2,
508 1 mM CaCl2), 0.03% Tween 20, and 0.01% BSA.

509 Wash Buffer: Prepared with 1% BSA, 0.2 U/μL RNase Inhibitor in PBS.

510 All buffers were pre-chilled on ice or at 4°C before use.

511 Frozen tissue samples were minced on dry ice and transferred to 1.5 mL microcentrifuge tubes,
512 with a sample volume not exceeding 500 μL.

513 500 μL of chilled Lysis Buffer was added to each sample. Tissues were homogenized on ice
514 using a Dounce homogenizer (Fisher 12-141-363) with 10-20 strokes over a 5-minute period.

515 An additional 500 μL of Lysis Buffer was added, and samples were incubated on ice for the
516 remainder of the 5-minute period, with intermittent mixing.

517 Homogenates were filtered through a 70 μm-strainer mesh, and the flow-through was collected
518 in a polystyrene round-bottom FACS tube.

519 The filtrate was transferred to a 1.5 mL tube, and 500 μL of Wash Buffer was added. Samples
520 were centrifuged at 500 g for 5 minutes at 4°C.

521 The supernatant was carefully removed, and the pellet was resuspended in 500 µL of Wash
522 Buffer. This washing step was repeated once.

523 After the final wash, nuclei were resuspended in Wash Buffer and counted. The suspension was
524 adjusted to a final concentration of 1000 nuclei/µL.

525 If necessary, an additional filtration step using a 40 µm Flowmi filter was performed to remove
526 any remaining debris.

527 Samples were kept on ice and then sent to the Single Cell Sequencing Core at the University of
528 Pittsburgh, targeting 8,000 cells for downstream sequencing.

529 **Fixed single nuclei extraction**

530 For FFPE single nuclei extraction, we used a modified version of the snPATHO-seq protocol
531 provided to us by the lab of Luciano Martelotto[28].

532 *Reagents and Equipment*

533 Reagents included Ethanol (Decon Laboratories #2701), Xylene (Epredia, 6601), Nuclease-
534 Free water (Invitraogen, AM9938), 1x Phosphate Buffer Saline (Ca2+ and Mg2+ free) (Corning,
535 21-031-CV), Liberase TM (Millipore Sigma, 5401119001), RPMI1640 (Gibco), 10% BSA (),TST
536 Buffer and Wash buffer (See above in Frozen Single Nuclei extraction). Equipment used
537 included a Thermomixer with adjustable shaking (Eppendorf) and a refrigerated centrifuge.

538 Nuclei Isolation Procedure

539 2-4 tissue sections (25 µm-thick) or punches were collected and stored at 4°C if not used
540 immediately.

541 Paraffin was removed by washing sections three times with 1 mL Xylene for 10 minutes each.
542 Samples were rehydrated through an ethanol gradient (100%, 70%, 50%, 30%) for 1 minute
543 each.

544 Samples were washed once with 1× PBS + 0.5 mM CaCl2.

545 Tissue digestion was performed in 1 mL RPMI1640 supplemented with Liberase TM (1 mg/mL),
546 for 60 minutes at 37°C with shaking at 800 rpm.

547 After digestion, 400 µL of TST Buffer was added, mixed, and centrifuged at 850 x g for 5
548 minutes at 4°C.

549 The pellet was resuspended in 250 µL TST buffer containing 2% BSA and 1 U/µL RNAse
550 Inhibitor, then homogenized using a Dounce homogenizer (10-20 strokes).

551 An additional 750 µL of the EZ Lysis buffer mixture was added, followed by further
552 disaggregation by pipetting up and down and incubation on ice for 5 minutes.

553 The sample was filtered through a 70 µm PluriStrainer and centrifuged at 850 x g for 5 minutes
554 at 4°C.

555 Nuclei were washed twice with wash buffer and resuspended in PBS 0.5x + 0.02% BSA, put on
556 ice and delivered to the Single Cell Sequencing Core or stored in cryopreservation (see below).

557 Samples were then processed for Chromium X run using Chromium Fix RNA Profiling (10x
558 Genomics) following the manufacturer's protocol.

559 For cryopreservation, samples were supplemented with Enhancer solution (10x Genomics) and
560 0,22um filtered 10% Glycerol provided by the Single Cell Sequencing Core, incubated on ice for
561 10 minutes, and stored at −80°C.

562 This method was optimized for the preparation of nuclei suspensions from formalin-fixed,
563 paraffin-embedded (FFPE) tissue samples for single-nucleus RNA sequencing applications.

564 **Patient Derived Xenograft Development**

565 Tumor chunks were excised from the organ, with a slice down the middle for increased media
566 perfusion. Samples were then shipped same day to Champion Oncology lab for implantation.
567 For cryopreserved tissue, chunks 0.5 cm cubed in size were excised and frozen in freezing
568 media (See Supplemental 1 for details). These were then shipped frozen to Champions
569 Oncology overnight when required.

570 **Organoid generation and culture**

571

572 Patient-derived organoids (PDOs) were generated from consented primary human breast
573 cancer tissue from the Pitt Biospecimen Core in accordance with Institutional Review Board
574 protocol STUDY22030183 by the Institute for Precision Medicine according to established
575 protocol (Sachs et al 2018), with the addition of β-estradiol to the medium. Briefly, tumors were
576 digested with collagenase (Sigma C9407) on a rotator, sheared, filtered, and embedded in
577 Cultrex RGF Basement Membrane Extract (R&D Systems™ 353301002) in 24-well non-treated

578 plates (Fisher 12-566-82). Media replaced every 2-3 days and PDOs passaged every 2-4
579 weeks.
580
581 **Organoid Growth Assay**
582 PDOs were dissociated using 0.25% trypsin, washed in Advanced DMEM/F12, seeded at
583 20,000 cells per well in 96-well round bottom plates (Corning 353227), and cultured in standard
584 growth media (250 ng/ml Recombinant Human R-Spondin-3, 5 nM Recombinant Human
585 Heregulin β-1, 5 ng/ml Recombinant Human KGF (FGF-7), 20 ng/ml Recombinant Human
586 FGF10, 5 ng/ml Recombinant Human EGF, 100 ng/ml Recombinant Human Noggin, 500 nM A
587 83-01, 5 mM Y-27632, 500 nM SB 202190, 1X B-27 Supplement, 1.25 mM N-Acetyl-L-cysteine,
588 5 mM Nicotinamide, 50 mg/ml Primocin, 10 mM HEPES, 1X GlutaMAX, 100 U/ml Antibiotic-
589 Antimycotic, and 1X Advanced DMEM).
590
591 **Computational methods**

592 Frozen single nuclei sequencing data were aligned using CellRanger 7.1.0 while Fixed Flex
593 single nuclei sequencing was aligned using Multiranger 7.1.0. Standard Seurat Recipe
594 Preprocessing was used and samples were then Harmony integrated for comparison. Celltype
595 assignments were done using GSEAPY CellMarker2024 followed by manual review.

596 **Data Access Statement**

597 All single-cell sequencing data will be made available on NCBI GEO at time of publication.

598 **Western Blot Methods**

599 Cellular protein lysates were harvested utilizing RIPA buffer (50mM Tris pH 7.4, 150mM NaCl,
600 1mM EDTA (Thermo Fisher Scientific #15-575-020), 0.5% Nonidet P-40 (Sigma Aldrich
601 #74385), 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1X HALT protease and
602 phosphatase cocktail (Thermo Fisher #78442). Samples were vortexed, probe sonicated for 15
603 seconds (20% amplitude, Ultrasonic Processor GEX130) and centrifuged at 14,000rpm at 4℃
604 for 15 minutes. Protein concentration was assessed using the Pierce Bicinchoninic acid (BCA)
605 protein assay (Thermo Fisher #23225). Unless otherwise stated, 50µg of each protein sample
606 was run on a 10% SDS-PAGE gel followed with a 90V transfer at 4℃ for 90 minutes to a PVDF
607 membrane (Millipore #IPFL00010). Membranes were blocked for one hour with Intercept PBS
608 blocking buffer (LiCor #927-40000) at room temperature with rocking. Antibody probing was
609 performed overnight at 4℃ with rocking: ERα, clone 60C (Millipore #04-820,

610 RRID:AB_1587018); HA (C29F4) (Cell Signaling Technologies #3724, RRID:AB_1549585); β-
611 actin (Millipore Sigma #A5441, RRID:AB_ 476744). After removal of primary antibodies, blots
612 were wash with 1X PBSTween 20 (0.1%) for 15 minutes, three times. Secondary antibodies
613 were applied for a one-hour room temperature incubation (1:10,000; anti-mouse 680LT (LiCor
614 #925-68020); anti-rabbit 800CW (LiCor #925-32211)). Imaging of membranes was performed
615 on the LiCor Odyssey CLx Imaging system.

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630 models for our program and look forward to continued advancements in model development.

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632 collaboration and advice, and we look forward to a continued expansion in the global community
633 of such programs to advance cancer research.

634 **Ethics Statement**

635 All research involving human participants was conducted with informed consent
636 obtained according to the ethical guidelines of the University of Pittsburgh Institutional
637 Review Board under STUDY19060376.

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649

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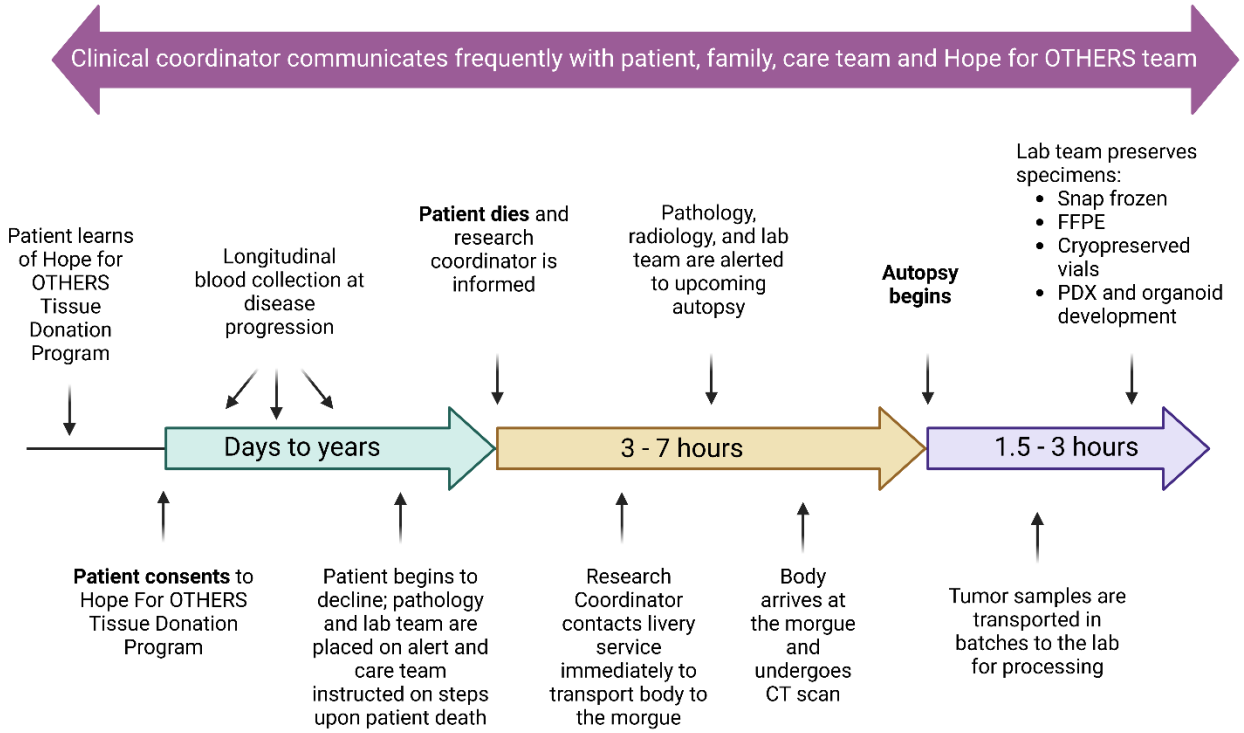
726

727

728 **Supplemental 1:**

729 A copy of our SOP for autopsy collection.

1 **Figure 1: Study design and workflow of the HFO program**



2

3 Figure 1: Diagram illustrating the study design and workflow of the HfO Tissue Donation
4 Program.

5

Figure 2: Summary of patients' treatment timelines and clinical details

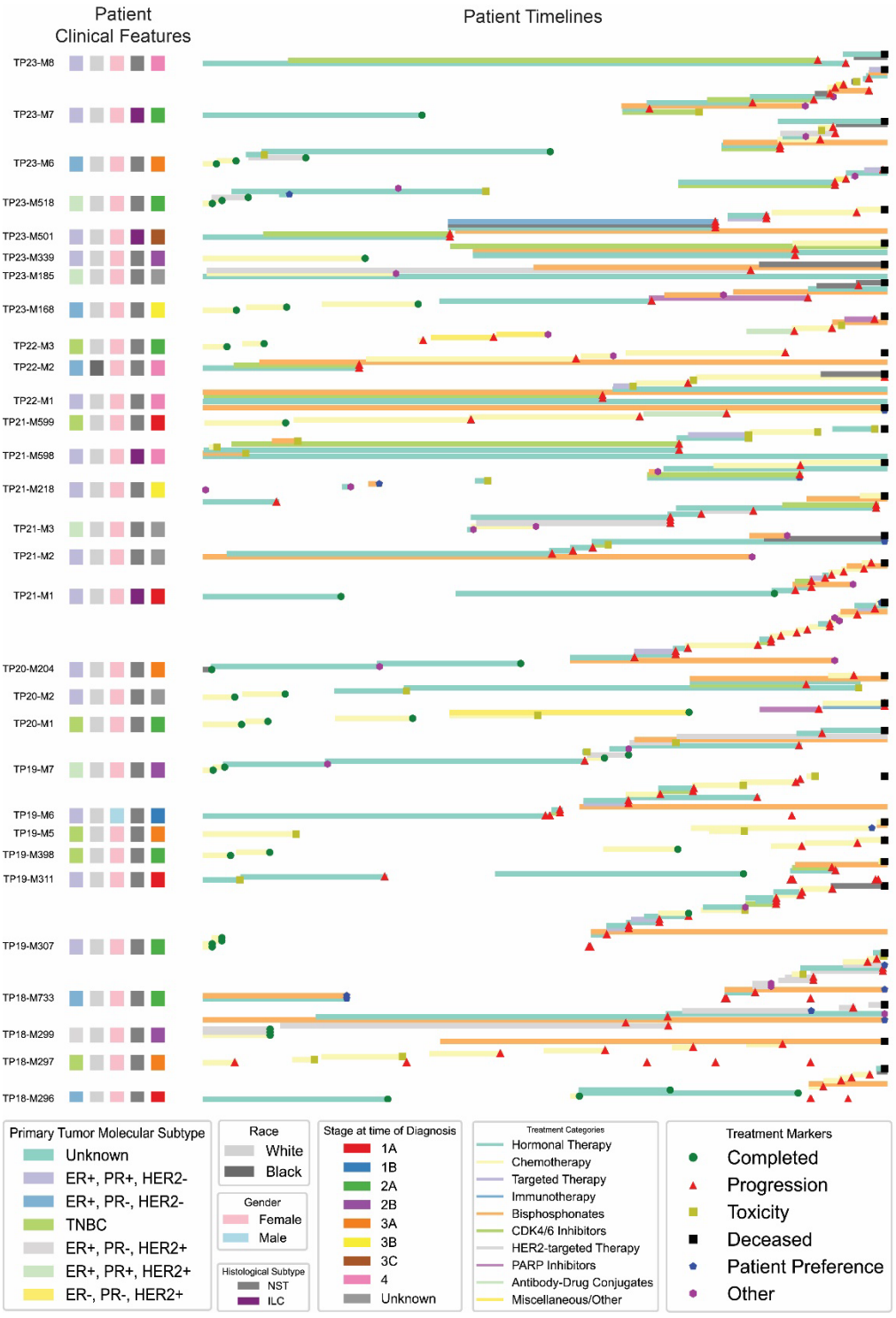


Figure 2 – Mixture line and normalized timeline chart showing a summary of HfO program, including primary tumor molecular subtype, race, gender, histological subtype, stage at time of diagnosis (pathological if available, clinical if not), treatment markers and treatment categories up to August 2024.

Figure 3: Examples of patient advocate media and figures demonstrating impact of key support staff.

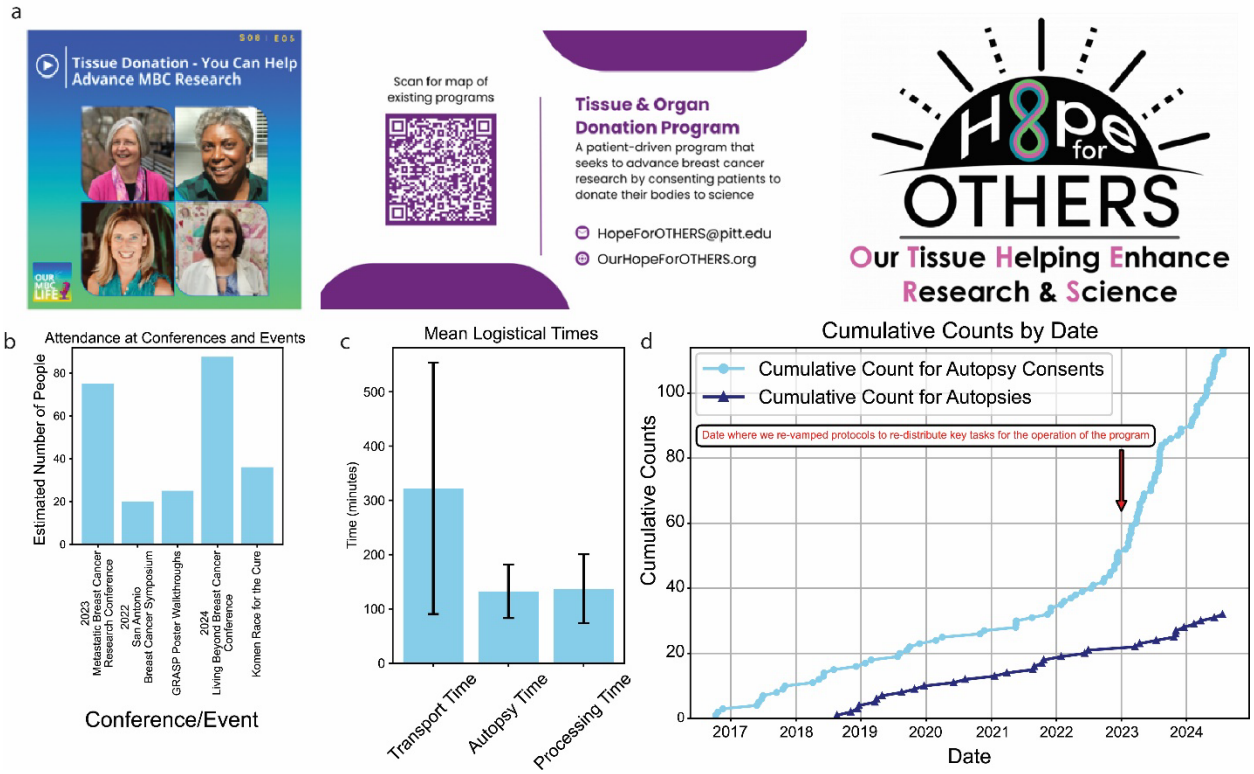


Figure 3: a. Promotion of tissue donation programs for advancing metastatic breast cancer (MBC) research via new media approaches such as podcasts, published media, and logo of the HfO program. Photos are of co-authors in this manuscript. **b**. Graph showing number of patients engaged at conferences/events. **c**. Bar plot shows the considerable standard deviation in mean transport time due to unique complexities within institutional and geographic contexts . **d**. Line graph shows the exponential increase in consent after an additional review of our operational protocols and rebranding (n = 114 consents, n = 34 autopsies [pre-2018 are not counted]). P-value from segmented regression 6.38E-31 for a change in consent rate slope post protocol review.

Figure 4: Summary of collected samples, and statistics on tissue diversity and counts in autopsy and non-autopsy settings.

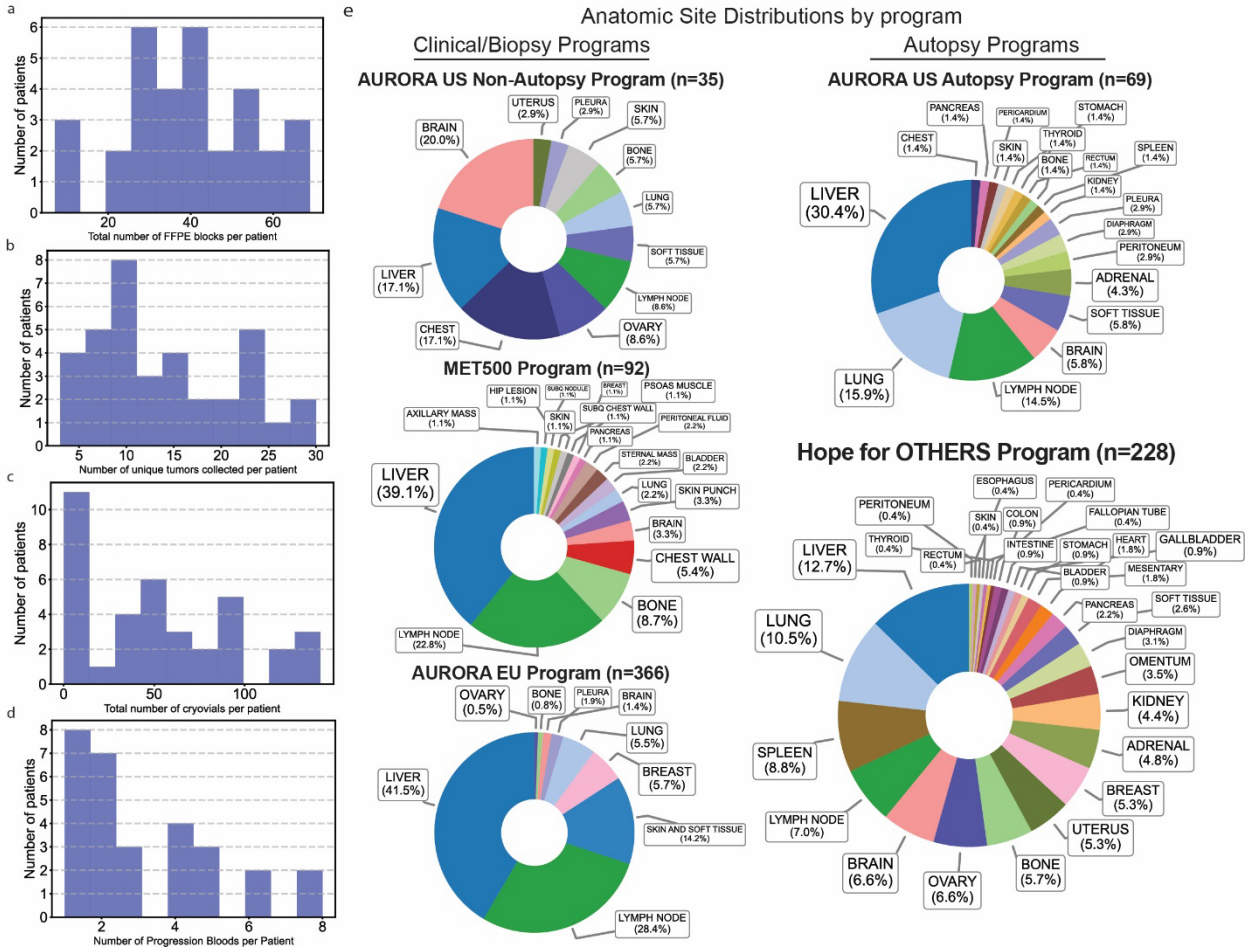


Figure 4: **a**. Histogram that summarizes statistics for FFPE. Total of 1244, median of 41, range of 7 to 69. **b**. Histogram that summarizes statistics for frozen tumors. Total of 511, median of 12, range of 3-27. **c**. Histogram that summarizes statistics for cryovials from autopsy. Total of 1952, median of 46 per patient, range from 0 to 142. **d**. Histogram that summarizes our longitudinal blood collections. **e**. Segment pie charts using data from the US Aurora, EU Aurora, MET500, and HfO reports showing the distinctly different range of tissues collected in autopsy and non-autopsy settings.

Figure 5: Diverse causes of death and subclinical metastases seen in autopsy settings, necessitating consistent collection.

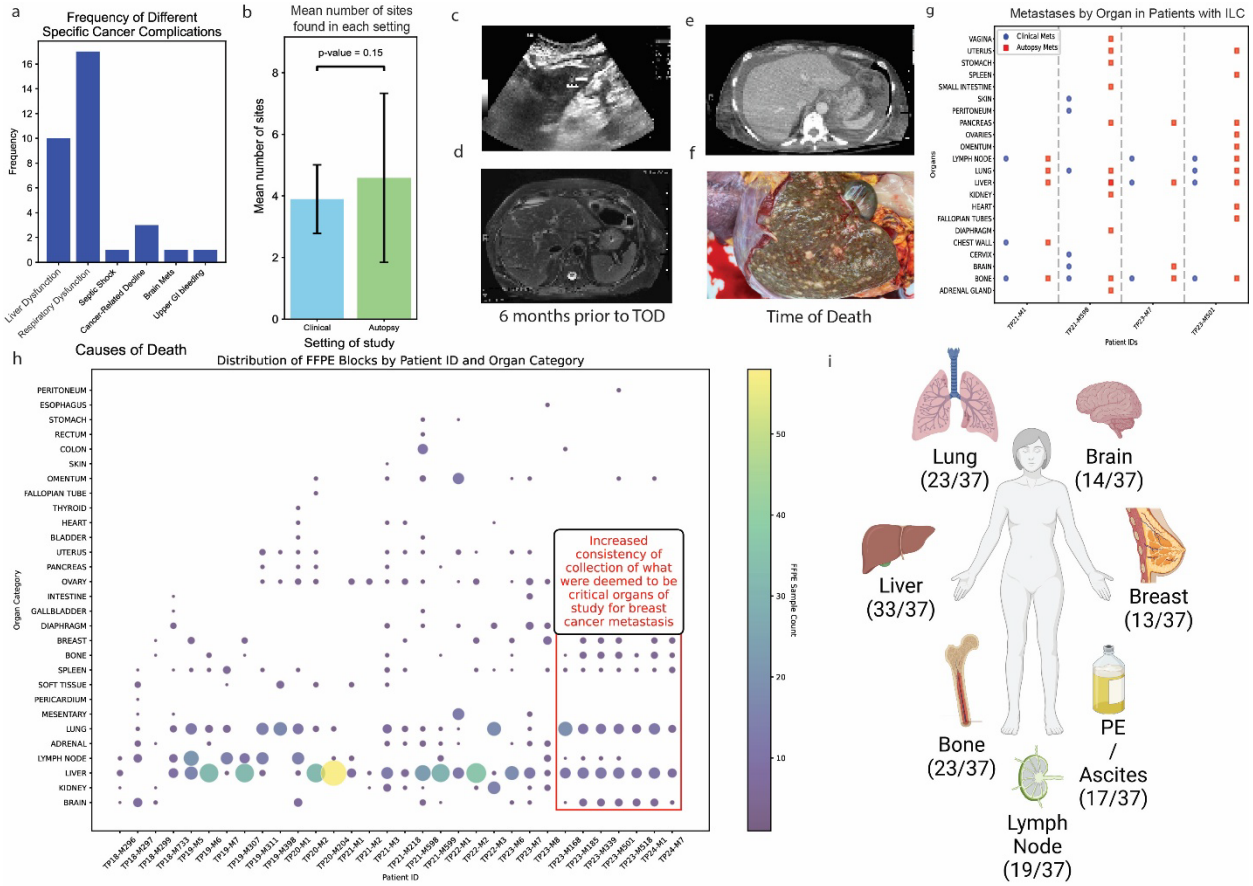


Figure 5: **a**. Bar plot showing the frequency of different causes of death in our program based on clinical note and autopsy report review (n = 37). **b**. Bar chart showing the mean increase in organs identified with metastases after careful pathological review on autopsy that were not identified in regular clinical monitoring, error bars are standard deviation. P-value 0.15 with paired t-test. Images of **c**. ultrasound and **d**. MRI in a patient with ILC showing the CT-undetectable liver metastases. **e**. CT image and **f**. autopsy image at time of death for the patient with ILC illustrating the discrepancy between a 'normal' CT and the organ status. **g**. Scatter plot showing the difference in metastases seen clinically and in autopsy for patients with ILC. Patients with ILC have much more spread in peritoneal tissues that are undetectable clinically. **h**. Scatter bubble plot showing our FFPE collection, red box highlights our improved protocol to increase consistency in grossly normal tissues. Color corresponds to size of bubble. **i**. Figure showing top 7 organ sites, collected whenever available, even if grossly normal under our revised protocol.

Figure 6: Single nuclei sequencing of paired frozen and fixed tissue samples.

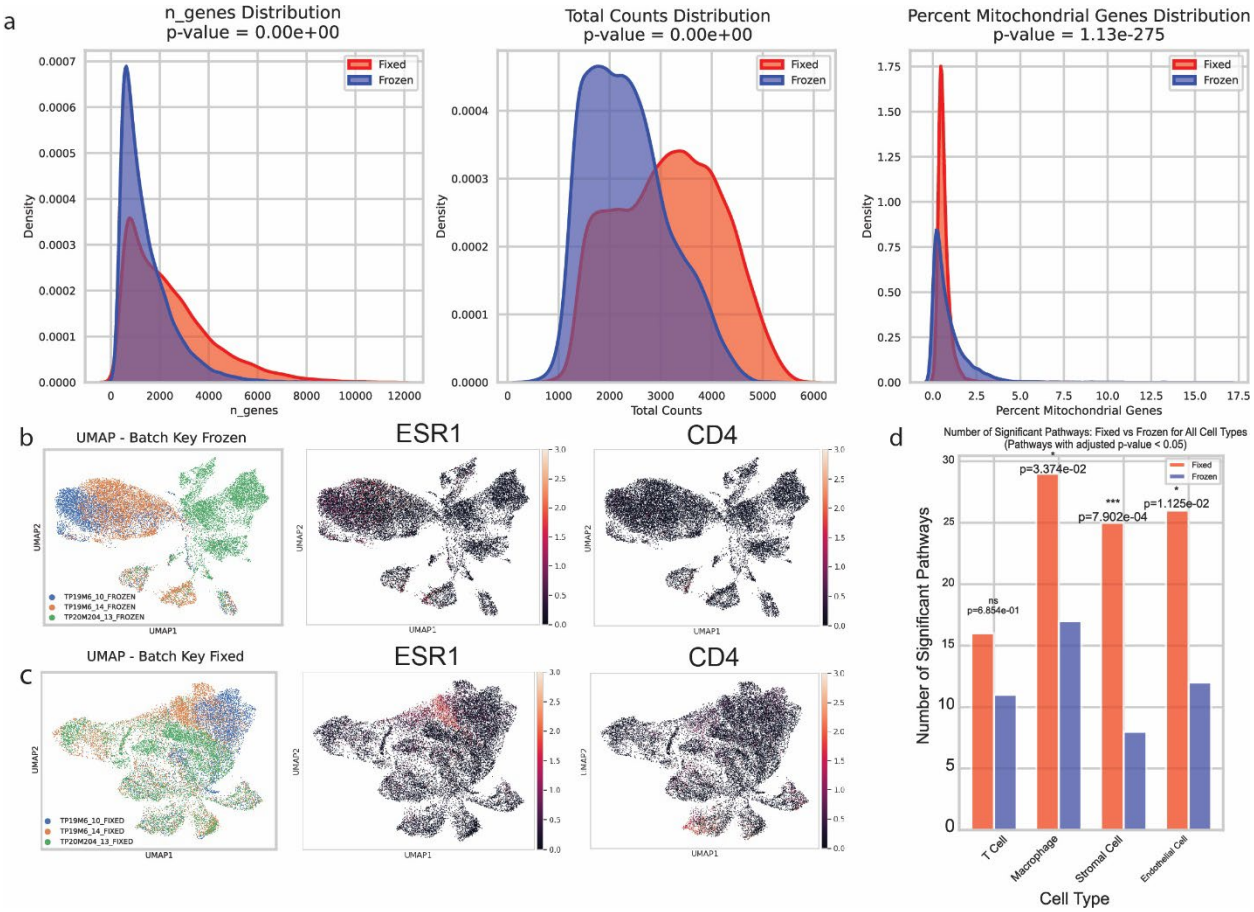


Figure 6: **a**. Side by side comparison of frozen vs fixed single nuclei sequencing data metrics shows significant improvement in fixed technologies with increase in genes detected, molecules detected, and less mitochondrial contamination. (n = 3) Run using 10x Genomics kit targeting 8000 cells. **b**. UMAP plot showing that frozen single nuclei sequencing lead to loss of signal causing failure of integration and removal of batch effects (n = 3), with diminished ESR1, and CD4 signal. **c**. Fixed single nuclei sequencing has better integration due to better signal recovery, with visible improvements in ESR1 and CD4 signal. PGR signal consistency across both sets shows that fixed technologies are not artificially introducing signal that isn't there. **d**. Bar plot showing the number of Hallmark pathways that have adjusted p-value less than 0.05 after pathway analysis between fixed and frozen cells, showing that fixed tissue almost always has better pathway signal. P-value calculated using chi-square test.

Figure 7: Logistic regression results for PDO and PDX generation success.

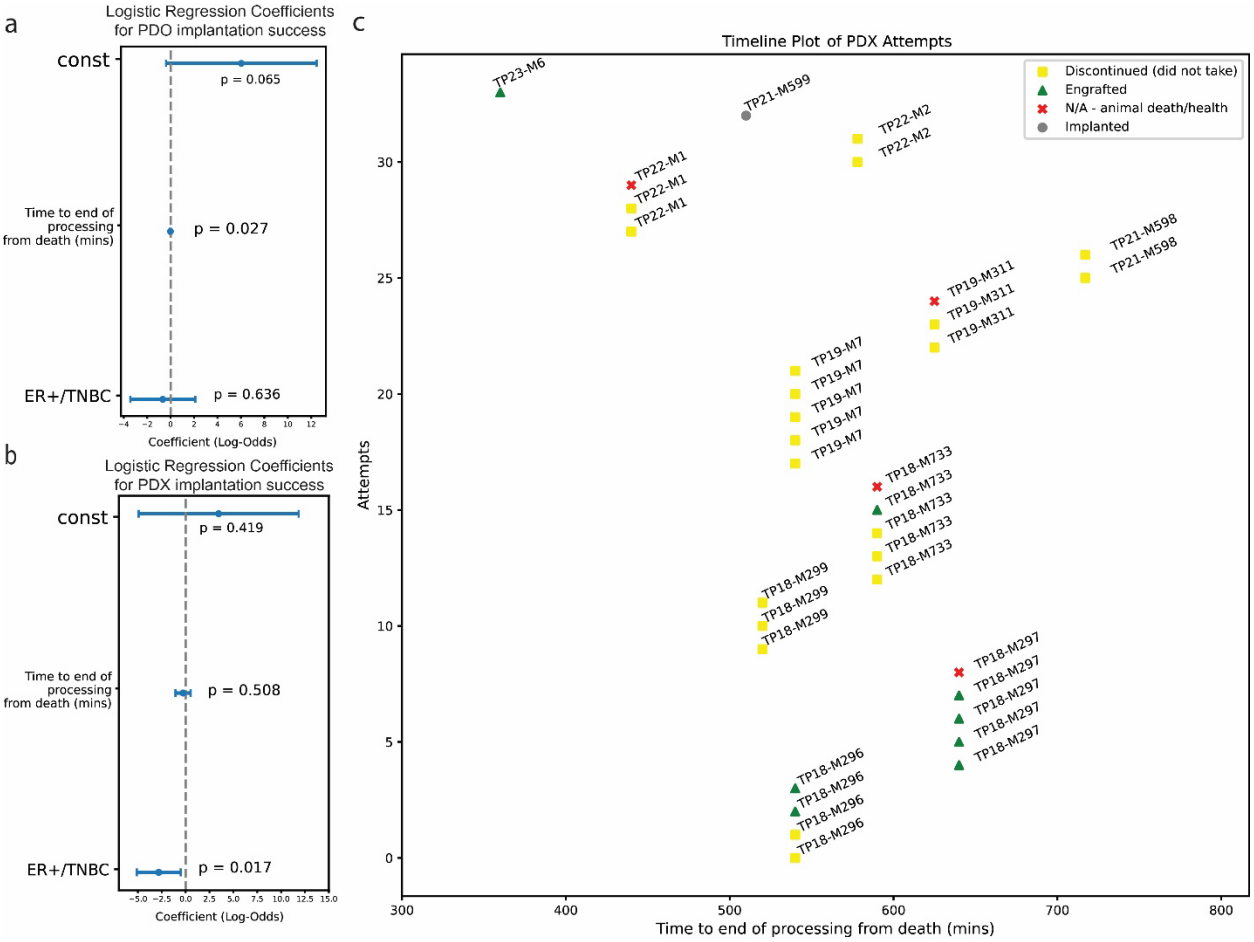


Figure 7: **a**. Logistic regressions for PDO attempts (n = 27 attempts) show that time to end of processing is predictive of success for PDO success. **b**. Logistic regressions for PDX attempts (n = 34 attempts) show that TNBC lesions are more likely to be successful. **c**. There are also significantly different probabilities of success on an intra-patient basis, implying that tissue of origin or tumor cellularity may play a role (n = 34).

Figure 8: Validation of expression of the ESR1-ARNT2 fusion detected in HfO samples.

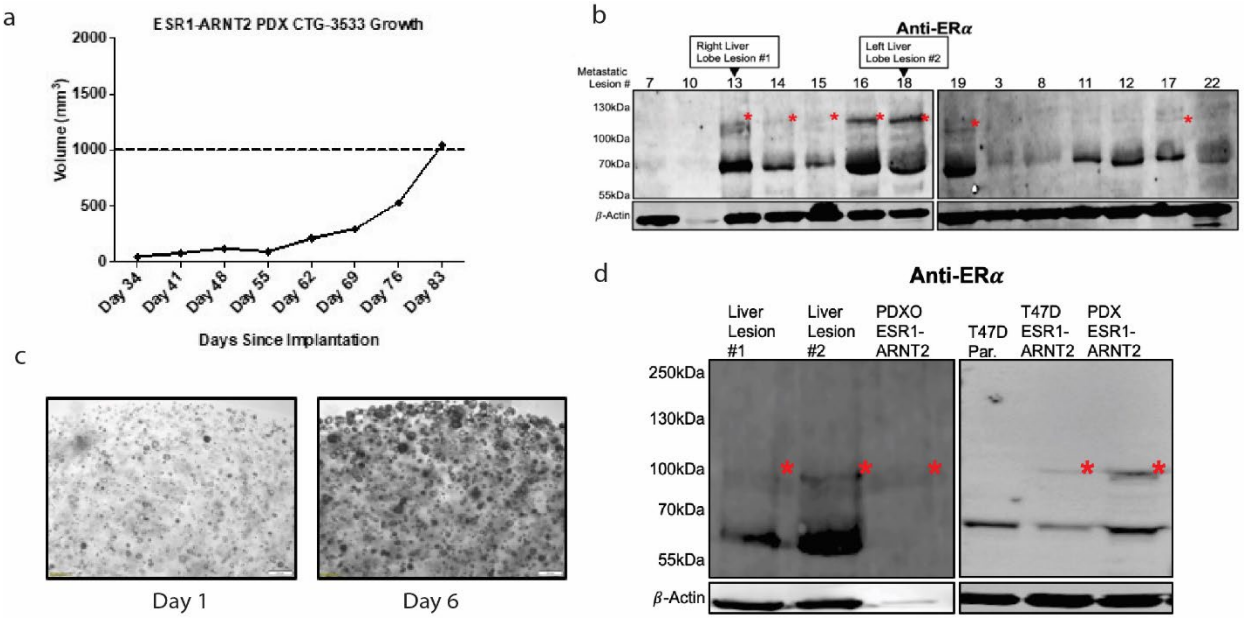
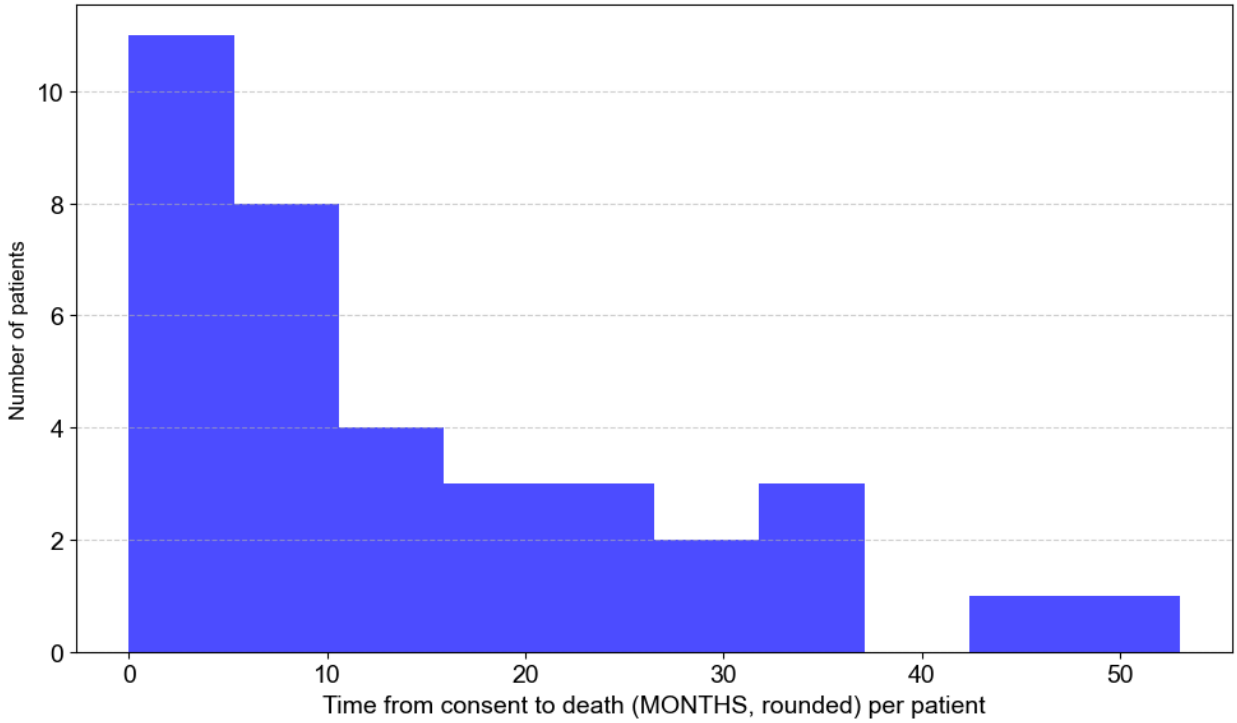


Figure 8: **a**. Growth of PDX model CTG-3533. **b**. ER-immunoblot of TP18-M733 metastatic lesion protein samples. Red asterisks denote the expected size for the ESR1-ARNT2 fusion protein. β-actin serves as loading control in both blots, n=1. **c**, PDXO from CTG-3533 PDX can be cultivated from the PDX to extend back to in vitro experimentation. **d**. PDXO shows low levels of expression of the fusion construct and heterogenous signal. Sequencing also allows design of the fusion construct to be transfected into cells lines like T47D, as seen on ER-western blot. Red asterisks denote the expected size for the ESR1-ARNT2 fusion protein. β-actin serves as loading control in both blots, n=1.

92 **Figure S1: Histogram for time from consent to death per patient.**



93
94 **Figure S1**: Histogram summarizing our time from consent to death in months, rounded.
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Figure S2: Pathway scores comparison between fixed and frozen tissue.

Figure S2. Downstream single cell analysis showing that fixed sequencing has better gene set percentage overlap and higher scores on average.

Figure S3: Illustration of difference between chunked and minced tissue.

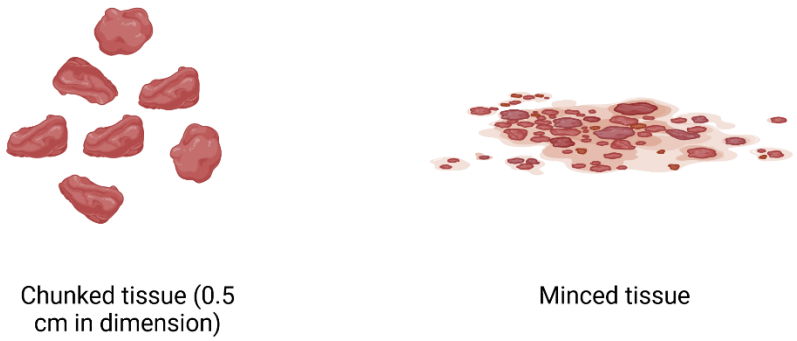

Figure S3. Illustration of the difference between chunked and minced tissue. Chunk tissues are preserved in 0.5cm – 1cm chunks. Minced tissues are chopped finely with a single-edged razor blade from an adjacent site.