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A model-based personalized cancer screening strategy for detecting early-stage tumors using blood-borne biomarkers

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Abstract

An effective cancer blood biomarker screening strategy must distinguish aggressive from non-aggressive tumors at an early, intervenable time. However, for blood-based strategies to be useful, the quality and quantity of the biomarker shed into the blood and its relationship to tumor growth or progression must be validated. To study how blood biomarker levels correlate with early-stage viable tumor growth in an mouse model of human cancer, we monitored early tumor growth of engineered human ovarian cancer cells (A2780) implanted orthotopically into nude mice. Biomarker shedding was monitored by serial blood sampling, while tumor viability and volume was monitored by bioluminescence imaging and ultrasound imaging. From these metrics we developed a mathematical model of cancer biomarker kinetics in different compartments that accounts for biomarker shedding from tumor and healthy cells, biomarker entry into vasculature, biomarker elimination from plasma and subject-specific tumor growth. We validated the model in a separate set of mice where subject-specific tumor growth rates were accurately predicted. To illustrate clinical translation of this strategy, we allometrically scaled model parameters from mouse to human and used parameters for PSA shedding and prostate cancer. In this manner, we found that blood biomarker sampling data alone was capable of enabling the detection and discrimination of simulated aggressive (2-month tumor doubling time) and non-aggressive (18-month tumor doubling time) tumors as early as 7.2 months and 8.9 years before clinical imaging, respectively. Our model and screening strategy offer broad impact in their applicability to any solid cancer and the biomarkers they shed, thereby allowing a distinction between aggressive vs. non-aggressive tumors using blood biomarker sampling data alone.

Keywords

Blood biomarkers; early cancer detection; mathematical model; personalized medicine; non-invasive imaging in animal models

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Quick Guide to Equations and Assumptions

Biomarker shedding model

We model biomarker shed (secreted or released) from tumor (cancerous) and healthy (non-cancerous) cells using a compartmental modeling approach based on the fundamental principles of mass balance. The model, biologically depicted in Fig. 3A, can be reduced to 2 compartments (Fig. 3C) in which $q_{pl}(t)$ is the amount (mass) of biomarker in whole-body plasma and $q_{periph}(t)$ is the amount (mass) of biomarker in a generalized “periphery” that accounts for all biomarker not measureable in a plasma sample. The peripheral compartment may either represent uptake or storage of biomarker into a physical region (e.g., interstitial or extravascular space) or a non-physical means of sequestering biomarker (e.g., binding or interaction with other proteins in plasma or extravascular space.)

The change in the amount of biomarker in a compartment with respect to time is the summation of the influx of biomarker from other sources (tumor cells, healthy cells, periphery) minus the elimination or efflux of biomarker from the compartment. For the 2-compartment model, the equations are:

$$\frac{dq_{pl}(t)}{dt} = u_t(t) + u_h(t) - \left(k_{el} + k_{periph,pl}\right)q_{pl}(t) + k_{pl,periph}q_{periph}(t)$$

$$\frac{dq_{periph}(t)}{dt} = k_{periph,pl}q_{pl}(t) - k_{pl,periph}q_{periph}(t)$$

where the influx of plasma biomarker from tumor cells is

$$u_t(t) = R_t N_t(t)$$

and the influx of plasma biomarker from healthy cells is

$$u_h(t) = R_h N_h(t) = u_{h,SS} = k_{el}q_{pl,SS}$$

and by definition of mass, $q_{pl,SS} \equiv c_{pl,SS} V_{pl}$.

Model parameters are described below. The derivation of the model steady state conditions in the absence of cancer is fully described in the Supplementary Materials.

Biomarker shedding model parameters

- k_{el} is the (irreversible) elimination rate of biomarker from plasma (day^{-1});
- $k_{pl,periph}$ is the rate of biomarker movement from periphery to plasma (day^{-1});
- $k_{periph,pl}$ is the rate of biomarker movement from plasma to periphery (day^{-1});
- R_t is the tumor cell biomarker shedding rate (ng/day/cell);

- $c_{pl,SS}$ is the steady-state plasma biomarker concentration in healthy subject (ng/ml);
- V_{pl} is the plasma volume of distribution (ml).

Tumor growth model

In cases for simulating early growth of sub-centimeter sized tumors, we used the monoexponential tumor growth model

$$N_t = N_{t0} e^{k_{gr} t}$$

with model parameters described below.

Tumor growth model parameters

- N_{t0} is the initial number of tumor cells at time $t=0$;
- k_{gr} is the rate of growth of the tumor cell population (day^{-1}).

Summary of Major Assumptions

1. Biomarker in each compartment is assumed well-mixed and homogeneous, thereby obeying compartmental model kinetics.
2. Tumor biomarker shedding rate R_t is the average shedding rate for all tumor cells of a given subject and is constant with respect to time and cell number. R_t is not a cell-specific shedding rate. It is assumed that R_t is initially characterized from an initial population of subjects (to determine a specific population mean and standard deviation) when the biomarker is being validated for early detection of a specific clinically relevant cancer(s).
3. The influx of tumor-shed biomarker is assumed proportional to the rate of tumor biomarker shedding R_t and the number of tumor cells $N_t(t)$, i.e., $u_t(t) = R_t N_t(t)$.
4. Healthy cells are defined to be any non-cancerous cells in any organ shedding the biomarker of interest. The number of biomarker-shedding healthy cells, $N_h(t)$, is generally unknown but assumed constant (i.e., cell proliferation and death rates are equal). The healthy cell biomarker shedding rate is assumed to be constant with respect to time and cell number, and therefore, the influx of healthy-shed biomarker is assumed constant and at steady state, i.e., $u_h(t) = R_h N_h(t) = u_{h,SS}$.
5. Biomarker may reversibly enter peripheral space and whole-body plasma.

A more detailed description of the model and its assumptions, validations and applications is provided in the Supplementary Materials.

Introduction

In cancer screening, early and accurate diagnoses are essential. It is critical to intercept aggressive tumors and effectively treat them while they are likely still localized to their

primary organ of origin, before they become more heterogeneous, unmanageable, and ultimately lethal. It is also important to minimize intervention on slow-growing tumors that may never pose a threat during the patient's lifetime, to reduce unnecessary stress and side effects of overtreatment. The health and well-being of a patient therefore depend on a medical team's ability to make timely and well-informed decisions regarding a patient's disease status.

Frequent monitoring of a patient's disease status is now becoming possible with inexpensive blood assays that measure levels of cancer biomarkers "shed" or released into the blood from newly developed tumors. However, even with repeated blood sampling to look for abnormal changes in cancer biomarker levels, a medical team still cannot make early, accurate decisions about disease outcome; a deeper quantitative understanding between the state of a tumor and the amount of biomarker in blood is needed. This correlation should include factors influencing blood measurements, such as biomarker pharmacokinetics (arrival into and elimination of biomarker from blood), the unknown number and aggressiveness of viable (live) tumor cells, and the accuracy of the blood assay.

Deriving and validating this mathematical relationship is further complicated by the lack of *in vivo* measurements for cancer blood biomarker shedding and early-stage tumor growth in human patient or animal models. Clinical data does not exist because: 1) for ethical, financial, and practical reasons, patients with lethal cancers cannot be serially monitored via blood and imaging over many years of untreated tumor growth; 2) many aggressive cancers are not discovered until they reach advanced stages, when early-stage biomarker shedding processes can no longer be properly studied; 3) to date, clinical cancer blood biomarkers are primarily utilized to monitor disease recurrence or treatment efficacy, and are still being fully validated for early-stage diagnosis. Without an understanding of the link between cancer blood biomarker levels and tumor growth in living subjects, it is not surprising that so far no one has been able to truly improve existing blood-based early cancer detection and screening strategies in patients.

Here, we demonstrate that the relationship between cancer blood biomarkers and tumor state may be exploited to screen for and monitor early cancers. We first establish that cancer biomarkers may serve as reporters of early tumor state in early cancer mouse models. We then develop a generalized mathematical model to map the link between a growing tumor and the biomarker levels shed into blood. Following validation of the mathematical model in mice, we then allometrically scale the model parameters from preclinical subjects (mice) to human patients to illustrate a new blood-based screening paradigm in which a patient's current blood biomarker sampling history can be used to classify the presence and aggressiveness of the disease (Supplementary Fig. S1). This yields a new personalized cancer screening methodology – one that leverages all existing blood data for a given patient and determines if the patient's blood history can cumulatively provide a useful prediction regarding that patient's cancer state or progression. The general mathematical model can be applied to any solid cancer and associated biomarker(s) shed. Importantly, the model can best be utilized in early clinical decision making to determine when to intervene on a potentially aggressive cancer, when to proceed with active surveillance of a non-aggressive

cancer, and how to optimally continue blood sampling in a specific patient whose disease state is unknown.

Materials and Methods

Cloning

We constructed a vector using a dual ubiquitin promoter approach to obtain high expression levels of both secreted alkaline phosphatase (SEAP) [1] and the bifusion optical imaging reporter firefly-luciferase-enhanced-green-fluorescent-protein (FL-eGFP) [2, 3]. First, the SEAP gene was PCR-amplified using a forward primer containing a *NheI* restriction enzyme site and a reverse primer with a FLAG tag sequence, stop codon, and *XhoI* restriction enzyme site. The restriction enzyme-digested product was cloned into a corresponding enzyme-digested mammalian expression vector (pcDNA 3.1(+)) downstream of a constitutive ubiquitin promoter (Ubi). The FLAG tag was incorporated, but not used here, to allow more sensitive pull-down analyses [4] in future studies. The gene for FL-eGFP was cloned into a separate mammalian expression vector (pcDNA 3.1(+)) under a constitutive Ubi promoter, and then re-cloned into the Ubi-SEAP-FLAG plasmid by PCR cloning using a forward primer flanking the 5' end of the ubiquitin promoter and a reverse primer flanking the 3' end of SV40-PolA, both with a *BglII* restriction enzyme site. The final plasmid (pcPur-Ubi-SEAP-FLAG-Ubi-FL-eGFP) was sequence confirmed (Sequetech) and then used in further experiments.

Cell Culture

All cells were cultured under sterile conditions, maintained in a 5% CO₂-humidified incubator at 37 °C, and tested regularly for the absence of mycoplasma contamination. Ovarian cancer cell line A2780 (epithelial ovarian carcinoma) was received in 2010, cultured in RPMI 1640 (Invitrogen) and authenticated via short tandem repeat (STR) profiling most recently in 2014 (Genetica DNA Laboratories). Human embryonic kidney cells (293T) were obtained from ATCC in 2010 and cultured in Dulbecco's modified Eagle's medium (Invitrogen). All cells were used within 6 months of authentication or resuscitation. All media were supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin (10000 U/ml) and 1% streptomycin (10000 µg/ml) solution (Invitrogen). Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Stable cell lines were obtained by antibiotic selection using 10 mg/ml puromycin (InvivoGen) in cell culture media. A2780 and 293T cells stably transfected with a single vector expressing SEAP-FLAG and FL-eGFP are denoted A2780-SEAP-FL-eGFP and 293T-SEAP-FL-eGFP, respectively.

The highest 1–5% of eGFP-expressing stable A2780-SEAP-FL-eGFP and 293T-SEAP-FL-eGFP cells were collected based on fluorescence activated cell sorting (FACS) using the FACS Aria (BD Biosciences). Collected cells were expanded and re-sorted two more times, and the highest SEAP and FL expressing single-cell clone was selected. These single-cell clones were used for all cell culture and animal experiments described in the manuscript.

SEAP and FL Correlation in Cell Culture

SEAP activity was measured and converted to concentration (ng/ml) using the Great EscAPE SEAP Chemiluminescence Assay 2.0 (Clontech). Cell culture shedding rates were calculated based on the mass of SEAP shed into 1 ml of media in 24 hr and normalized by the number of plated cells. To show that non-transfected cells do not shed detectable levels of SEAP, SEAP levels were compared in 10% FBS media (without cells) and 10% FBS media collected from plated non-transfected cells. Both samples lacked cellular production of reporter proteins (SEAP and FL), but exhibited a minimal background signal attributed to the assay itself (i.e., background optical signal).

To examine the correlation between SEAP and FL expression in cell culture, between 1×10^2 and 1×10^6 A2780-SEAP-FL-eGFP or 293T-SEAP-FL-eGFP cells were plated in 1 ml media in 12-well plates. After 24 hr, media was removed and centrifuged at 12,000 rpm for 1 min, and 25 μ l of the media supernatant was stored at -80°C to be assayed for SEAP activity later. Next, 0.5 ml of D-Luciferin (10 μ g/ml in PBS) was added to each well and FL activity was measured in the IVIS Lumina Imaging System (PerkinElmer) under sequence luminescence scan mode using 5–300 sec acquisitions. FL activity was quantified in terms of total flux (photon/s), average radiance (photon/s/cm²/sr) and maximum radiance (photon/s/cm²/sr) using the Living Image 3.2 software (PerkinElmer). In wells containing at least 1×10^3 A2780-SEAP-FL-eGFP cells, the correlation between cell number and media SEAP concentration (Fig. 1D, unpaired two-tailed $P=0.005$), and FL average radiance (Fig. 1F, unpaired two-tailed $P=0.0001$), was significantly higher relative to non-transfected A2780 cells. Notably, the correlation between media SEAP concentration and FL activity was also high for 1×10^3 to 1×10^6 plated A2780-SEAP-FL-eGFP cells (Fig. 1G, $R^2=0.99$).

Tumor Implantation, Blood Sampling, and Imaging of Living Mice

All animal experiments were performed in accordance with a protocol approved by the Stanford University Administrative Panels on Laboratory Animal Care (APLAC). Female nude mice (4–5 weeks old) were purchased from Charles River Laboratories. Cells were collected by trypsinization at 70–80% confluence, washed in media, resuspended and counted in PBS. Three extra aliquots of cells were prepared to manually validate approximate cell number using a hemocytometer.

Mice were anesthetized with 2% isoflurane in oxygen at 2 l/min. For orthotopic implantation of ovarian cancer cells in the ovary, surgery was performed under aseptic conditions. A longitudinal paramedian skin incision was made on the left flank, followed by a small incision in the abdominal muscle that was enlarged by blunt dissection. The left ovarian fat pad and ovary were retracted through the incision and a small tear was made in the bursal membrane to expose the infundibulum. Bleeding was minimized by preemptively applying a small drop ($< 10 \mu$ l) of epinephrine (1:100,000) to the bursal membrane. A single injection of either 3×10^5 , 3×10^4 , 3×10^3 or 3×10^2 A2780-SEAP-FL-eGFP cells or 3×10^5 A2780 cells in a volume of $< 15 \mu$ l PBS was delivered into the ovarian bursa under a dissection microscope using a 31.5-gauge insulin syringe. The needle was inserted into the oviduct near the infundibulum, toward the bursa and ovary. The incision was stitched with absorbable sutures and the skin was stapled closed with autoclips. We chose a 15-day

preclinical endpoint for “early” detectable ovarian tumors based on the *in vivo* ultrasound measurements (2–4 mm diameter [5], which includes healthy ovarian tissue).

For subcutaneous implantation of 293T cells, either 1×10^5 293T cells or 1×10^5 , 5×10^4 , 1×10^4 , 5×10^3 , 1×10^3 or 5×10^2 293T-SEAP-FL-eGFP cells were prepared in 25 μ l PBS and 25 μ l growth-factor reduced Matrigel (BD Biosciences) and implanted dorsally and 1 cm anterior to the tail.

Tumor dimensions were measured by digital caliper (Fisher Scientific) and/or using the Vevo 2100 high-resolution ultrasound micro-imaging system (VisualSonics). Mice bearing fewer than 3×10^3 cells did not develop SEAP-detectable or FL-detectable tumors (Figs. 1I and 1J), and no tumors were found in these animals using *in vivo* ultrasound imaging or during post-experiment dissection.

Plasma samples (25–30 μ l) were obtained by submandibular bleeding at various times post-tumor implantation using 5-mm Goldenrod Animal Lancets (Medipoint, Inc.) and BD Lithium Heparin-Coated Microtainer plasma separation tubes (Fisher Scientific) following the manufacturers’ recommended protocols. All plasma samples were stored at -80°C until assayed.

To quantify FL bioluminescence activity, mice were anesthetized with 2% isoflurane in oxygen at 2 l/min and D-luciferin (3 mg in 100 μ l PBS per mouse) was administered intraperitoneally. Mice were imaged in the IVIS Spectrum (PerkinElmer) under sequence luminescence scan mode using 5–300 sec acquisitions. Living Image 3.2 software was used to quantify tumor FL in terms of average radiance (photon/s/cm²/sr).

To measure the degradation (or irreversible elimination) of SEAP from mouse plasma, we injected either 900 ng or 5 ng SEAP intravenously via the tail vein and SEAP concentration was measured in plasma samples taken at various times ranging from 1 min to 10 days post-injection.

Mathematical Model of Cancer Biomarker Shedding and Tumor Growth

We developed a series of compartmental models (Figs. 3B and 3C) that account for biomarker shedding by tumor and healthy cells, based on the biological depiction shown in Fig. 3A. Here, healthy cells are defined to be any non-cancerous cells in any organ shedding the biomarker of interest. Biomarker produced in tumor cells and healthy cells is shed into whole-body plasma (q_p) and may reversibly enter the tumor extravascular space ($q_{t\text{Periph}}$) or healthy extravascular space ($q_{h\text{Periph}}$). Biomarker in each compartment is assumed well-mixed and homogenous, thereby obeying compartmental model kinetics. The influx, $u_t(t)$, of tumor-shed biomarker is assumed proportional to the rate of tumor biomarker shedding, R_t (ng/day/cell), and the number of tumor cells, $N_t(t)$, i.e., $u_t(t) = R_t N_t(t)$.

Healthy cells are also assumed to shed biomarker at a constant rate, R_h (ng/day/cell). The number of biomarker-shedding healthy cells, $N_h(t)$, is generally unknown but assumed constant (i.e., cell proliferation and death rates are equal). Therefore the influx, $u_h(t)$, of healthy-shed biomarker is constant and at steady state, i.e., $u_h(t) = R_h N_h(t) = u_{h,SS}$.

Biomarker movement from compartment q_i to q_j is represented by fractional rate constant $k_{j,i}$ (day^{-1}). Irreversible elimination of biomarker from plasma occurs at rate k_{el} (day^{-1}), which accounts for degradation or removal of intact biomarker from plasma.

Supplementary Tables S1 and S2 summarize the model state variables and parameters. For the derivation of the model equations at steady state in the absence of cancer and additional model details, see Supplementary Materials.

Reducing the 3-Compartment Model to a 2-Compartment Model

The 3-compartment model has “tumor peripheral” ($q_{t\text{Periph}}$) and “healthy peripheral” ($q_{h\text{Periph}}$) compartments to account for biomarker unavailable for measurement from plasma (Fig. 3B). Because we aimed to model the growth of early tumors prior to the development of an established vascular network and well-defined tumor microenvironment [6], the 3-compartment model can be further reduced to two compartments (Fig. 3C), in which a generalized “periphery” (q_{periph}) accounts for all biomarker not measurable in a plasma sample. The peripheral compartment accounts for the uptake or storage of biomarker into non-plasma space. We note that while the peripheral compartments may represent a physical region (e.g., interstitial or extravascular space), they may also represent a non-physical means of sequestering biomarker (e.g., binding or interaction with other proteins in plasma or extravascular space).

Model Fitting and Parameter Estimation Methodology

All plasma SEAP data was $\log_{10}$ -transformed so that the rescaled data measurements appeared uniformly on the same axes and could be uniformly weighted (i.e., each data point d_i was assigned a weight $w_i=1$) during the fitting. We used the simulation, analysis and modeling software SAAM II (The Epsilon Group) [7] for all model fitting and sensitivity analyses. Model fits were compared using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), with lower AIC and BIC values indicating a better fit [8, 9].

FL average radiance ($FL(t)$) was converted to viable tumor cell number ($N_t(t)$) using the equation, $\log_{10}[N_t(t)] = (\log_{10}[FL(t)] - 0.80)/1.06$, which is the equation of the line fitted through the A2780-SEAP-FL-eGFP data in Fig. 1L. For each tumor-bearing mouse, the calculated $N_t(t)$ was used as a linearly interpolated forcing function in the tumor biomarker input function, $u_t(t) = R_t N_t(t)$. The 2-compartment model (Fig. 3C) was simultaneously fitted to individual tumor-bearing mouse plasma SEAP data (Figs. 3E–G) and to the mean plasma SEAP levels in healthy mice following intravenous injection of 900 ng or 4 ng SEAP (Fig. 3D) to estimate the following parameters: 1) R_t , the tumor cell biomarker shedding rate (ng/day/cell); 2) k_{el} , the elimination rate of biomarker from plasma (day^{-1}); 3) $k_{pl,\text{periph}}$, the rate of movement of biomarker from periphery to plasma (day^{-1}); 4) $k_{\text{periph},pl}$, the rate of movement of biomarker from plasma to periphery (day^{-1}); 5) $c_{pl,ss}$, the steady-state plasma biomarker concentration in healthy subject; and 6) V_{pl} , the plasma volume of distribution (ml).

Validation of the 2-Compartment Model Using Training and Prediction Data Sets

Any blood biomarker being advocated for cancer detection utility in the clinic must first be evaluated in a given population of patients; once the biomarker pharmacokinetics have been determined in this “training set,” the ability of the biomarker to predict cancer progression may be determined in a separate patient population, the “prediction set.” The utility of the mathematical model was established by showing that the estimated training set parameters can provide decent predictions of cancer progression in the prediction set. Here, we used *in vivo* preclinical data obtained for plasma SEAP levels and tumor volume determined by bioluminescence imaging to explicitly validate of the model’s predictive capability by successfully estimating tumor growth parameters in a prediction subset of subjects. An analogous study can be performed for other cancers and biomarkers of interest, once such data becomes available; this is of particular interest and need in the clinical setting.

Mice were randomized into training and prediction subsets ($n=9$ mice per subset). Randomization trials were repeated 10 times. For each randomization trial, the 2-compartment model was fitted simultaneously to all plasma SEAP data in the training subset to obtain population parameter estimates for R_t , V_{pl} , $c_{pl,SS}$, k_{el} , $k_{pl,periph}$ and $k_{periph,pl}$, which define SEAP shedding rates and pharmacokinetics (Supplementary Table S4). These population parameter estimates were then used to predict individualized tumor growth parameters N_{t0} and k_{gr} in individual mice in the prediction subset (Supplementary Fig. S5). In this simplified example, early-stage tumor growth was estimated using the monoexponential equation,

$$N_t(t) = N_{t0} e^{k_{gr} t},$$

where N_{t0} is the initial number of tumor cells implanted and k_{gr} is the growth rate of the tumor cell population. We used the monoexponential equation here to obtain, in the simplest sense, a basic generalization of tumor presence and progression (e.g., $N_{t0} > 0$, $k_{gr} > 0$). This is not unreasonable, as early-stage preclinical xenograft models have been shown to follow monoexponential (or variants such as Gompertzian) growth [10, 11]. We do not attempt to model tumor growth or cancer development on a multiscale level, as has been done in other mathematical models incorporating several tumor cell states (hypoxic, dormant, etc.) and biological processes (angiogenesis, apoptosis, senescence, etc.) [12], although these models may theoretically be substituted in place of the monoexponential equation in future studies.

Results

Cancer-Specific Blood Biomarkers Correlate with Early-Stage Tumor Viability

Because no clinical biomarker has yet proven successful for early cancer screening purposes [13, 14], and since the potential of blood biomarker screening for early cancers has not been validated yet in human patients [14, 15], a critical first step is to verify that cancer biomarkers are capable of detecting small (sub-centimeter) early-stage tumors in living animals. We do this here by first developing tumorigenic cell lines distinguishable from endogenous healthy cells based on their ability to be highly detectable via the blood and

imaging. We stably transfected human epithelial ovarian cancer cell line A2780 with two tumor-specific reporter genes: 1) secreted alkaline phosphatase (SEAP) [1], a truncated form of human placental alkaline phosphatase not normally produced in any cell and thus shed only from transfected tumor cells; and 2) the bifusion optical imaging reporter, firefly luciferase fused with enhanced green fluorescent protein (FL-eGFP) [2, 3], which enables both sensitive detection of cell viability via bioluminescence imaging (BLI) and cell sorting capabilities via fluorescence imaging (Fig. 1A). Use of a tumor-specific biomarker and sensitive imaging modality necessarily reduces a biologically complicated biomarker shedding system into an ideal one in which biomarker is knowingly released only from imageable (quantifiable) tumor cells. Here, the stably transfected A2780-SEAP-FL-eGFP clone highly expressing SEAP and FL-eGFP shed 1,600-fold more SEAP (Fig. 1B) and expressed 42,000-fold higher FL average radiance (Fig. 1C) relative to non-transfected A2780 cells in culture. This enabled simultaneous and highly sensitive detection via shed biomarker (Fig. 1D) and cellular imaging (Figs. 1E and 1F), with both biomarker and imaging reporters correlating well with respect to cell number and each other, over at least 3 log-orders of magnitude, for as few as 1×10^3 cells ($R^2=0.99$, Fig. 1G).

It has been reported that BLI signal correlates well with viable transfected cell number in culture and with large tumor burden in various subcutaneous cancer xenograft models [16–20], but not for sub-millimeter early-stage solid tumors implanted orthotopically, let alone for deep-tissue cancers such as ovarian carcinoma, which are even more difficult to detect via optical methods due to increased light attenuation. To examine how blood biomarker kinetics change over the course of early viable tumor growth *in vivo*, we surgically implanted a range of A2780-SEAP-FL-eGFP cells (from 3×10^2 to 3×10^6 cells) orthotopically in the nude mouse ovary and monitored tumor growth via ultrasound imaging (Fig. 1H), blood SEAP (Fig. 1I) and BLI signal (Figs. 1J and 1K and Supplementary Fig. S2) for at least 15 days of “early” tumor growth, while the ultrasound images showed that the healthy and tumor tissue in the ovary was less than 5 mm in diameter. FL activity correlated well with implanted cell number as early as 4 hours post-implantation for a range of 3×10^4 to 3×10^6 implanted cells ($R^2=0.98$, Fig. 1L). We observed a high correlation between FL average radiance and plasma SEAP concentration for groups of mice bearing 3×10^4 , 3×10^5 , or 3×10^6 A2780-SEAP-FL-eGFP cells ($R^2=0.62$ – 0.79 , Fig. 1M), and a higher correlation for the combined data of all replicate mice ($R^2=0.86$, Fig. 1M). Not surprisingly, these overall correlative trends were also observed in individual mice (Fig. 2), even in mice that remained healthy for observation up to 25 days. This indicates that without confounding background biomarker shedding from healthy cells, the correlation between tumor-shed biomarker and tumor cell viability is notably high, and remains so possibly beyond the 15-day “early growth” period. Finally, to examine the correlation in other cell lines and xenograft models, we repeated the transfection and SEAP-FL correlation analyses in the human embryonic kidney cell line 293T in culture and in a subcutaneous xenograft mouse model using a stably-transfected 293T-SEAP-FL-eGFP clone. The correlation between plasma biomarker levels and tumor viability was also consistently high *in vivo* ($R^2=0.91$, Supplementary Fig. S3).

We found that as few as 3×10^4 ovarian cancer cells were detectable by SEAP shed into the 2-ml mouse blood volume. A rough extrapolation to patients suggests that 9×10^7 cells may

lead to detectable biomarker levels in 6 liters of patient blood volume, assuming equivalent biomarker shedding rates and a linear scale-up for humans. Given a 1×10^6 cells/mm³ tumor density [21, 22], this corresponds to a tumor volume of about 90 mm³ (or 4.5³ mm³) which may be detectable by a clinical biomarker such as CA125, if CA125 were shed at 500-fold higher rates than currently measured [21].

Because frequent blood-based and imaging-based early cancer monitoring cannot practically be performed in human patients, this manipulatable living mouse model of early cancer growth is advantageous because: 1) a known, low number of cancer cells can be monitored with high sensitivity and specificity via a tumor-specific secreted biomarker; 2) tumor growth and viability can be easily localized and quantified via non-invasive imaging methods in a living subject, over the natural course of cancer progression. Although an *in vivo* biological correlation exists between cancer blood biomarker levels and tumor size in both orthotopic (Fig. 1M) and subcutaneous xenograft mouse models (Supplementary Fig. S3J), the mathematical relationship between these blood and tumor measurements has not yet been established and validated *in vivo*. This quantitative link is important for understanding why SEAP exemplifies an ideal early cancer biomarker, and for evaluating how endogenous clinically relevant biomarkers may be better exploited to detect aggressive tumors earlier.

***In Vivo* Cancer Biomarker Shedding Rate Measurable For Individual Subjects**

Most clinical cancer blood biomarkers are shed not only from tumor cells, but from healthy (non-cancerous) cells in benign conditions as well. Therefore, while an exclusively tumor-shed biomarker such as SEAP may be used to detect an early-stage tumor, two natural follow-up questions are: 1) what are the *in vivo* shedding rates of an ideal, exclusively tumor-shed biomarker that enable early tumor detectability, and 2) how does background biomarker shedding from healthy cells interfere with tumor detection *in vivo*?

To derive the dynamic *in vivo* relationships between biomarker shedding, plasma biomarker kinetics and tumor growth, we next developed a general mathematical model to describe biomarker rates of change in three *in vivo* compartments: tumor extravascular space, healthy extravascular space and whole-body plasma (Figs. 3A and 3B, Supplementary Tables S1 and S2). The general 3-compartment model can be extended to virtually any solid tumor and associated biomarkers shed, and is intended to be further tailored for a specific cancer and biomarker or biomarker panel. During early tumor growth, before leaky tumor vasculature has been established and biomarker is still entering healthy vasculature, the 3-compartment model reduces to 2 compartments (Fig. 3C). To our knowledge, these models are the first to characterize the *in vivo* shedding rates, vascular permeabilities and blood pharmacokinetics of a given biomarker of interest, relative to the presence of early-stage cancer *in vivo*.

For example, to characterize SEAP in the mice bearing orthotopic A2780-SEAP-FL-eGFP ovarian tumors, we first measured the half-life of SEAP in blood in healthy mice following intravenous injection of purified SEAP (Fig. 3D), and then simultaneously fitted the 2-compartment model equations (Supplementary Materials) to this data and to each tumor-bearing mouse's 15-day blood SEAP profile (Figs. 3E–G). The model provides *in vivo* estimates for the following parameters: 1) R_t , the tumor cell biomarker shedding rate

(ng/day/cell); 2) k_{el} , the elimination rate of biomarker from plasma (day^{-1}); 3) $k_{pl,periph}$, the rate of movement of biomarker from periphery to plasma (day^{-1}); 4) $k_{periph,pl}$, the rate of movement of biomarker from plasma to periphery (day^{-1}); 5) $c_{pl,ss}$, the steady-state plasma biomarker concentration in a healthy animal (ng/ml); and 6) V_{pl} , the plasma volume of distribution (ml) (Supplementary Table S3).

A major advance of this mathematical modeling analysis is that an *in vivo* biomarker shedding rate, R_t , can now be quantified for an individual living subject, relative to any early tumor burden present. This rate represents an average rate for a given time period and inherently takes into account any subject-specific biological differences in tumor cell biomarker expression and tumor growth rate. For the ovarian cancer mouse model, SEAP shedding rate R_t values ranged from 2×10^{-5} to 8×10^{-5} ng/cell/day, consistent with the shedding rate measured in cell culture conditions (6×10^{-5} ng/cell/day, Supplementary Fig. S4). Interestingly, mice implanted with 3×10^4 A2780-SEAP-FL cells exhibited roughly 4-fold lower shedding rates compare with mice in the 3×10^5 and 3×10^6 cell groups; this is consistent with the higher probability of initial cell death (or limited cell survival) for these extremely few implanted cells.

The mathematical model is intended to predict subject-specific (personalized) tumor growth rates based on a subject's cancer blood biomarker profile alone. To validate the model structure, we used biomarker and tumor growth data from a randomized initial population of mice to train the model and estimate biomarker shedding rates and other pharmacokinetic parameters (Supplementary Fig. S5, Supplementary Table S4). These parameters were then used to make subject-specific predictions about tumor growth in a separate population of mice (Supplementary Fig. S6). Using SEAP as an example biomarker, the model was used to accurately predict the tumor growth profiles of individual mice based on their plasma SEAP data alone. Similar integrative experimental and mathematical modeling studies can be performed for validation of cancer biomarkers in the clinic, once the appropriate serial blood and tumor data becomes readily available.

Model Extends to Clinical Cancer Biomarkers Shed From Tumor and Healthy Cells

SEAP was chosen as an ideal cancer biomarker because it is exclusively tumor-shed and therefore enabled gold-standard *in vivo* blood detectability. To determine how background biomarker shedding from healthy cells may interfere with tumor detection for other biomarkers *in vivo*, as is common for clinical cancer biomarkers, and to mimic other biological influences on plasma biomarker levels (e.g., autoantibodies that decrease biomarker levels, changes in vascular permeability, etc.), we next examined a range of parameter values that may be exhibited by more realistic clinical biomarkers. We performed a 1-way sensitivity analysis by re-simulating plasma SEAP levels following a 10-, 100- or 1000-fold increase or decrease in a single population parameter estimate (Fig. 4). Clearly, increasing R_t would increase the predicted plasma SEAP levels (Fig. 4A) and prompt earlier detectability. Altering k_{el} , $k_{pl,periph}$, and $k_{periph,pl}$, however, have little impact on plasma SEAP levels (Figs. 4B–D). Decreasing V_{pl} , the apparent plasma volume of distribution, would theoretically increase plasma SEAP levels, although physically this may not be feasible (Fig. 4E). Interestingly, model simulations showed that increasing $c_{pl,ss}$ 1000-fold,

as may be expected if SEAP were produced endogenously by healthy cells, would obscure detectability of this early ovarian tumor even beyond 15 days (Fig. 4F).

Mathematical Model Establishes Blood-Based Personalized Early Cancer Screening Paradigm

Previous cancer biomarker mathematical models have used blood biomarker levels to estimate tumor size, which is one measure of the state of a tumor [21, 22]. A more useful metric is the aggressiveness of the cancer, which may indicate how quickly the disease is progressing. To illustrate the translational impact of the model on patient screening and early diagnosis of aggressive and non-aggressive cancers in the clinic, we next present a simulated scenario in which asymptomatic patients are actively monitored for a specific blood biomarker indicative of the presence of a potentially lethal cancer (Supplementary Materials). Here, we aim to identify patients who unknowingly have aggressive primary tumors, using their plasma biomarker measurements alone, at an early, interventionable time before the cancer becomes large enough ($>5^3 \text{ mm}^3$) to be likely detectable by current clinical imaging modalities such as MRI, PET, CT or ultrasound. We also aim to identify patients that have non-aggressive or slow-growing cancers that may not pose a threat for several decades (if at all), to minimize their risk of being overtreated.

There is currently no clinical data available tracking blood biomarker levels and tumor volume measurements in individual patients over the course of untreated tumor growth. Acquisition of such data is not trivial, requiring population-based screening studies spanning decades of effort, thousands of highly monitored cancer patients with varying degrees of disease, and immense financial resources to enable frequent blood screening and imaging. To illustrate the potential of our modeling strategy, we therefore allometrically scaled [23–25] the biomarker parameters estimated from our *in vivo* ovarian mouse model to patients and forward-simulated clinical tumor growth and corresponding plasma biomarker sampling profiles for patients undergoing regular blood draws (e.g., every 3 months, although any sampling frequency can be implemented) (Supplementary Materials). To mimic common obstacles hindering blood-based early cancer detection, we assumed biomarker shedding from both tumor and healthy cells, at rates similar to the well-studied clinical blood biomarker PSA (Supplementary Materials). We also incorporated assay measurement error (technical variability), which would affect a clinician's ability to accurately interpret biological anomalies (Supplementary Materials). We summarize our findings here in three representative patients who have either: 1) aggressive disease, requiring intervention within 12 months of detection (Figs. 5A–D and Supplementary Figs. S7A–D); 2) moderately aggressive disease, requiring intervention within 1–5 years (Supplementary Figs. S8A–C); or 3) non-aggressive disease, requiring intervention after 5 years, if at all (Figs. 6A–C).

We use the mathematical model of blood biomarker shedding to introduce a new modeling-based screening strategy that integrates the patient's blood sampling history into the decision-making process for blood-based diagnosis. After each blood draw, the biomarker shedding model is fitted to the patient's cumulative blood biomarker data. If the patient's blood sampling history suggests the potential presence of a growing tumor, the model provides an estimate of the current tumor burden volume as well as a measure of the

aggressiveness of the cancer (in terms of the time required for the tumor to become imaging-detectable), with a specified “threshold of uncertainty” (a reflection of the model’s goodness of fit, based on the standard deviation of the tumor growth rate estimated during the model fitting). A screening decision can be made when the model fits the data within an acceptable threshold of uncertainty, which can be tailored for each individual patient (e.g., here, we use a 20% coefficient of variation in the estimated tumor growth rate). The aim of the following simulations is to illustrate a “dynamic” mathematical modeling strategy that may be exploited to better design optimal blood sampling schedules based on the specific parameters of a given patient. The goal of this strategy is to utilize a patient’s cumulative blood sampling history (current and past measurements) to determine whether the patient should proceed to imaging or other diagnostic follow-up.

Fig. 5A illustrates the simulated blood sampling history (red circles) and true but unknown tumor growth curve (blue circles) of an example patient who unknowingly has an aggressive cancer (2-month doubling time). Although blood samples are acquired every 3 months, the tumor remains small and does not become imaging-detectable until month 49.1 at its earliest (when it reaches a volume of 5^3 mm^3 , Fig. 5A); even then, the tumor may go undetected until it becomes symptomatic or otherwise clinically apparent. Without a quantitative framework to link plasma biomarker levels to tumor size, the patient’s plasma biomarker are assumed “normal” through month 51, when the tumor is 6.24^3 mm^3 and finally large enough to be imaging detectable. But based on the patient’s blood sampling history alone, could it have been detected earlier, and known that it would grow aggressively?

For the representative patient shown in Fig. 5, at least 11 blood samples are needed for the model to initially detect the presence of a tumor (month 30, Fig. 5B). At this time, the model predicts the tumor will not become imaging-detectable until month 46.0 ± 132.3 , and since the variability in this prediction is greater than the 20% threshold of uncertainty ($132.3/46.0 \times 100 = 288\%$), this suggests that more blood samples are needed to hone the model predictions for this patient and distinguish the cancer from random fluctuations in assay measurement error. By including at least 3–4 more blood samples (Figs. 5C and 5D), the variability reduces to 21% by month 39 and then 7% by month 42, when the model predicts that the tumor will become imaging detectable in 7.2 months, indicative of aggressive tumor growth. In this case, the model predicts the tumor is only $2.21^3 \pm 0.74^3 \text{ mm}^3$ based on the patient’s blood data alone. We note that inclusion of more samples taken every 3 months, which would require additional waiting time, would further improve the variability of the parameter estimates but at the potential increased risk of the patient’s health (Fig. 5A). Instead, an alternative patient-specific sampling schedule can be implemented in which additional blood samples are taken immediately and more frequently (daily or weekly) as soon as the model detects the presence of cancer (e.g., starting as early as month 30 for the patient in Fig. 5B); with this additional data, it is less likely that the blood sampling history will be a misrepresentation of the true tumor growth. Importantly, the mathematical model is intended to be refitted to all biomarker data after every blood sample, to assess the likelihood of aggressive disease relative to any new biomarker data incorporated into the patient’s blood sampling history.

Because variability of the biomarker sampling data can affect the accuracy and confidence (certainty) of the model predictions, we next sought to quantify how the model predictions of detection time and tumor burden volume would change in the absence of assay measurement error (Supplementary Materials). Supplementary Figs. S7A–D illustrate the true biomarker data (with $\mu_b=0$ and $\sigma_b=0$) from the representative patient in Fig. 5. We first note that measurement error does not affect the accuracy (predicted imaging detection time = month 49.1) or certainty (1%) of the model predictions when all blood samples are included for this patient (Supplementary Fig. S7A vs. Fig. 5A). The model's confidence in its predicted detection time via imaging is lower in the presence of measurement error (288% vs. 1076%) because, in this case, the random noise incorrectly suggests the presence of a more aggressive tumor. Again, this highlights the need to immediately acquire more frequent blood samples, particularly when a reasonable (20%) threshold of uncertainty is exceeded. As noted in all cases (Fig. 5 and Supplementary Fig. S7), the certainty of the model predictions improves as more blood samples are incorporated into the fitting (from 288% to 1% in Fig. 5A–B, and from 1076% to 1% in Supplementary Fig. S7A–B).

To illustrate how this modeling-based screening strategy may be implemented over years of blood sampling, we repeated the modeling analysis in patients representing moderately aggressive disease (Supplementary Figs. S8A–C) and non-aggressive disease (Figs. 6A–C). In each case, respectively, the model was able to predict the presence of disease 2.5 and 8.9 years earlier than imaging, suggesting that these patients were not at immediate risk for aggressive disease (<1 year).

We note that in all cases examined, the predicted detection time (and thus the aggressiveness of disease) was estimable with a greater certainty than the predicted tumor diameter. This is important because although additional blood samples may be needed to pinpoint the size of the tumor, the aggressiveness of the tumor can be estimated with better certainty early on.

Discussion

This new comprehensive set of *in vivo* plasma biomarker profiling data enables a more rigorous, kinetic analysis in individual living subjects, over the entire unperturbed course of early cancer progression. Importantly, this integrative experimental-modeling approach can be extended to characterize new classes of candidate clinical biomarkers released indirectly from tumors – including circulating nucleic acids [26], autoantibodies generated in response to tumor-associated antigens, [27–33], and synthetic biomarkers shed following exogenous administration of gene vector [34] or nanoparticle-based agents [35] – which exhibit widely varying blood kinetics, half-lives, and assay diagnostics. Future studies may also broaden the applications of this patient-specific mathematical modeling approach beyond early cancer screening to address other unmet clinical needs, such as monitoring and predicting cancer treatment response. Additional studies in living animals and in patients may also be pursued to better understand biomarker shedding mechanisms during *in vivo* tumor development. In particular, genetically engineered mouse models can be used to mimic spontaneous cancer growth and immune interactions within the tumor microenvironment. The kinetics of biomarker shedding in healthy patients as a function of age, genetics and environmental

factors may eventually be incorporated into the mathematical model as the relevant patient-specific and population data becomes available.

The advancement of new technologies that minimize technical (measurement) variability may further improve the accuracy of the model predictions. More sensitive blood-based measurements are now becoming possible, especially for newer classes of biomarkers such as circulating tumor DNA [36–38]. Weekly or daily monitoring of biomarker levels is becoming possible with the development of new wearable or implantable diagnostics, such as the glucose monitoring contact lens [39]. These improvements in the accuracy and frequency of cancer biomarker sampling may require a decade or more of development, but can be developed in parallel with the validation of better early cancer biomarkers, alongside the implementation of the personalized blood screening schedules developed here, to facilitate the modeling-based classification of aggressive and non-aggressive cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Major Findings

We showed that blood biomarkers highly shed from early-stage cancers in living subjects may serve as feasible reporters of tumor cell viability and disease aggressiveness. We developed a new mathematical modeling-based personalized cancer screening strategy to aid in early clinical decision making to determine when to intervene on a potentially aggressive cancer, when to proceed with active surveillance of a non-aggressive cancer, and how to optimally continue blood sampling in a patient whose disease state is unknown.

Fig. 1. Plasma biomarker levels correlate with tumor viability *in vivo* in an orthotopic early ovarian cancer mouse model

(A) Plasmid pcPur-Ubi-SEAP-FLAG-Ubi-FL-eGFP used to highly co-express correlated levels of tumor-specific biomarker SEAP and bioluminescence imaging bifusion reporter FL-eGFP. (B) Comparison of shed SEAP levels and (C) FL activity (total flux, p/s (red); average radiance, p/s/cm²/sr, (blue); maximum radiance, p/s/cm²/sr, (green)) in stably-transfected A2780-SEAP-FL-eGFP single cell clones in cell culture 24 hr after plating (mean±SEM shown for *n*=3 wells per clone). (D) Media SEAP concentration and (E, F) FL activity were assessed 24 hours after plating A2780-SEAP-FL-eGFP clone 1 cells (closed circles) or non-transfected A2780 cells (open circles) (mean±SEM shown for *n*=4 wells per

cell number). (G) The correlation between FL average radiance and media SEAP concentration for the indicated number of plated cells ($n=4$ wells per cell number). Dashed lines indicate signal due to media alone (in absence of cells), indicating inherent assay background signal not due to production of reporter proteins (SEAP or FL). (H) Detection of ovarian tumors via *in vivo* ultrasound imaging in one representative mouse with 3×10^4 A2780-SEAP-FL-eGFP cells implanted in the left ovary. (I) Plasma SEAP and (J, K) *in vivo* FL average radiance were monitored in mice inoculated with 3×10^6 (red, $n=6$ mice), 3×10^5 (yellow, $n=6$ mice), 3×10^4 (blue, $n=6$ mice), 3×10^3 (green, $n=4$ mice), 3×10^2 (purple, $n=4$ mice) A2780-SEAP-FL-eGFP cells or 3×10^5 non-transfected A2780 cells (white, $n=3$ mice). Plasma SEAP concentration and FL average radiance were indistinguishable from baseline (healthy) mouse levels for 3×10^2 and 3×10^3 A2780-SEAP-FL-eGFP cells and 3×10^5 A2780 cells. (L) *In vivo* FL activity measured within 4 hr of cell implantation correlated well with implanted cell number for the indicated n replicates. (M) The correlation between plasma SEAP and *in vivo* FL activity was plotted at each time point for mice bearing 3×10^6 (red, $n=6$ mice), 3×10^5 (yellow, $n=6$ mice) or 3×10^4 (blue, $n=6$ mice) A2780-SEAP-FL-eGFP cells. Solid black line indicates overall correlation in all measurements for $n=18$ A2780-SEAP-FL-eGFP tumor-bearing mice.

Fig. 2. Correlation between plasma SEAP concentration and FL average radiance for individual mice

Mice bearing either 3×10^6 (red, $n=6$ mice), 3×10^5 (yellow, $n=6$ mice) or 3×10^4 (blue, $n=6$ mice) A2780-SEAP-FL-eGFP cells in the ovary.

Fig. 3. Mathematical model of cancer biomarker shedding and tumor growth

(A) Biological schematic illustrating biomarker influx from tumor cells, $u_t(t)$, and from healthy cells, $u_h(t)$. Fractional rate constants $k_{i,j}$ represent transfer of biomarker from compartment j to compartment i . See Supplementary Tables S1 and S2 for a description of all model state variables, parameters and units. **(B)** Mathematical schematic of the general 3-compartment model, which reduces to **(C)** a 2-compartment submodel. **(D)** Plasma SEAP concentration was measured at various times following intravenous injection of 900 ng (solid line) or 5 ng SEAP (dashed line) in healthy mice (without tumor). Model fits from all replicate mice are similar and therefore appear superimposed. For tumor-bearing mice,

plasma SEAP levels were monitored following implantation of **(E)** 3×10^6 , **(F)** 3×10^5 or **(G)** 3×10^4 A2780-SEAP-FL-eGFP cells in the ovary in $n=6$ replicate mice per group. Solid lines in **(E)**, **(F)** and **(G)** indicate model fits to each mouse replicate, fitted simultaneously with data in **(D)**.

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Fig. 4. One-way sensitivity analyses to compare SEAP to other potential cancer biomarkers
 Parameter estimates were averaged for the $n=6$ mice from the 3×10^4 A2780-SEAP-FL-eGFP cell group. Plasma SEAP levels were then simulated after a 0-fold (black —), 10-fold (blue —), 100-fold (blue - - -) or 1000-fold (blue •••) increase or decrease in the indicated parameter estimates: (A) R_t ; (B) k_{el} ; (C) $k_{pl,periph}$; (D) $k_{periph,pl}$; (E) V_{pl} ; (F) $c_{pl,ss}$.

Fig. 5. Application of the mathematical model to personalized screening of patients with asymptomatic aggressive disease

Simulated screening scenario in which patient plasma biomarker levels were sampled every 3 months during the simulated growth of an aggressive tumor (2-month doubling time). The model was fitted (red curve, left y-axis) to simulated patient blood biomarker sampling data (red circles, left y-axis) alone for (A) the entire 51-month blood sampling history until the tumor became imaging detectable, or up to (B) 30 months, (C) 39 months, or (D) 42 months. The model-predicted tumor growth curve (blue curve, right y-axis) was then compared to the true simulated tumor growth data (blue circles, right y-axis) not used in model fitting. The model-predicted parameters (mean $\pm$ SD) included t_{image} , the estimated time of imaging

detectability, and d_{biom} , the estimated tumor diameter at the last biomarker sample. Yellow region indicates the lead time gained when the model predicts the imaging-detectable time within a threshold of 20% uncertainty. Gray region indicates the time period after the tumor becomes imaging-detectable. Note differences in left y-axis scale between panels.

Fig. 6. Application of the mathematical model to personalized screening of patients with asymptomatic non-aggressive disease

Simulated screening scenario in which patient plasma biomarker levels were sampled every 3 months during the simulated growth of a non-aggressive tumor (18-month doubling time). The model was fitted (red curve, left y-axis) to simulated patient biomarker sampling data (red circles, left y-axis) alone for (A) the entire 37-year blood sampling history until the tumor became imaging detectable, or up to (B) 27.25 years, or (C) 28 years. The model-predicted tumor growth curve (blue curve, right y-axis) was then compared to the true simulated tumor growth data (blue circles, right y-axis) not used in model fitting. The model-predicted parameters (mean $\pm$ SD) included t_{image} , the estimated time of imaging detectability, and d_{biom} , the estimated tumor diameter at the last biomarker sample. Yellow region indicates the lead time gained when the model predicts the imaging detectable time

within a threshold of 20% uncertainty. Gray region indicates the time period after the tumor becomes imaging detectable. Note differences in left y-axis scale between panels.

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