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A Statistical Test of the Hypothesis that Polyclonal Intestinal Tumors Arise by Random Collision of Initiated Clones

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Summary. The random collision hypothesis is a mathematical idealization of intestinal tumor formation that can account for the polyclonal origin of tumors without requiring a mechanistic description of clonal interaction. Using data from recent polyclonality studies in mice, we develop a statistical procedure to test the random collision hypothesis. Elements from stochastic geometry and approximations due to Armitage (1949, *Biometrika* **36,** 257–266) support a statistical model of tumor count data. Bayesian analysis yields the posterior distribution of the number of heterotypic tumors, from which p-values are computed to test random collision.

Key words: Cancer biology; Conditional predictive p-value; Markov chain Monte Carlo; Random geometric graph.

1. Background

Usually DNA is replicated without error during mitosis; however, mutations and other damage can occur that transform a normal cell to an aberrant state. The intestinal epithelium is one of the most proliferative tissues; ample opportunity exists for the damage of sufficient significance to initiate tumor growth. A tumor has a monoclonal origin if its constituent cells all trace by descent to a single ancestral cell that is aberrant relative to the normal tissue. Otherwise the tumor has a polyclonal origin. In spite of considerable research, the dynamics of tumor initiation and early tumor growth in the intestine have been difficult to measure and remain poorly understood (Garcia et al., 1999; Shih et al., 2001; Preston et al., 2003).

Studies in mice (Griffiths et al., 1989; Merritt, Gould, and Dove, 1997; Thliveris et al., 2005) and humans (Novelli et al., 1996, 2003) provide evidence for the polyclonal origin of at least some intestinal tumors. The idea behind these lineage-marker studies is that any intestinal cell assumes one of two distinguishable marker types, and, further, that the type of a cell is passed faithfully to its descendant cells (i.e., to its derived clone). For example in studies by Merritt et al. (1997) and Thliveris et al. (2005), each mouse was generated by fusing an embryo carrying the ROSA26 transgene to an embryo lacking this marker. The intestinal surface of each chimeric animal was a patchwork of blue ($ROSA^+$) and white ($ROSA^-$) cells. A polyclonal tumor forming on the border between blue and white patches had the opportunity to be heterotypic; a polyclonal tumor that originated in the center of a patch far from cells of the other type would have been homotypic. In binary lineage-marker studies, any heterotypic tumor is polyclonal, but a polyclonal tumor is not necessarily heterotypic. Because it assumes one of only two types, a binary marker is unable to fully resolve all the different clones that may be present in a single tumor. In spite of this limitation and others, clonality studies yield useful information on the origin of intestinal tumors, providing definitive evidence that at least some intestinal tumors have a polyclonal origin.

The biological mechanisms that generate polyclonality are not well understood. Unlike the standard paradigm for monoclonal tumors (e.g., Nowell, 1976), the polyclonal tumor cannot have a simple history in which a series of uncorrected mutations accumulate in the cellular descendants of a single aberrant cell. Some kind of interaction among distinct clones may be required. For example, an initiated clone might emit molecules that transform neighboring normal cells into neoplastic cells which then contribute to the tumor mass. Alternatively, initiated clones might be at a selective disadvantage in the tissue unless they are in close proximity to other initiated clones. Various scenarios requiring the interaction of initiated aberrant clones may explain polyclonality, but there is an elementary possibility that does not require any form of active interaction. It is *random collision*. Random collision refers to the possibility that polyclonality is a consequence of independent random initiation events, which, having occurred by chance in sufficiently close proximity, result in a neoplasm recognized as a single tumor. Though it is a mathematical idealization, random collision serves as a useful null hypothesis by which various tumor models can be gauged. None of the evidence presented prior to Thliveris et al. (2005) could clearly refute random collision as a parsimonious explanation of polyclonal tumor formation, in part because the multiplicity of intestinal tumors was too high.

Here we more fully develop the statistical test that was introduced and used in Thliveris et al. (2005) to test the random

Table 1
Data from Thliveris et al. and posterior inference: Tumor count columns indicate the total number of tumors in the small intestine broken down according to tumor phenotype. The last column indicates the conditional predictive p-value computed under the random collision model predicting the number of heterotypic tumors, and using collision distance $\delta = 1.5$ mm.

		Tumor count					
Mouse ID	Blue tissue (%)	Total	White	Blue	Heterotypic	NA	p-value
100	20	19	6	5	5	3	0.000
122	85	24	6	13	3	2	0.002
154	20	9	5	2	2	0	0.002
209	60	19	10	2	3	4	0.004
225	30	24	21	0	2	1	0.062
237	50	9	3	2	2	2	0.004
244	40	8	3	0	5	0	0.000

collision hypothesis. Data are available on tumor multiplicities, sizes, and phenotypes (heterotypic/homotypic) from the binary lineage-marker study of Thliveris et al. (2005), with tumor multiplicity and phenotype data from the seven chimeric mice being shown in Table 1. Briefly, both contributing embryos in each of these chimeric mice carried a mutation in the Apc gene, predisposing the animal to intestinal tumor growth. Tumors were counted throughout the small intestine after the animals were sacrificed just after 60 days of age. Tumor phenotypes were examined histologically by multiple pathologists to be confident that each assessment was accurate. Tumors were relatively small. On average the maximum diameter was 0.85 mm with standard deviation 0.5 mm; all but one of the tumors had maximum diameter smaller than 2.0 mm. A striking feature of the data in Table 1 is the high fraction of heterotypic tumors (22%) among those with an unambiguous phenotype. Considering the low tumor multiplicity and the relatively small tumor sizes in these mice, one expects collisions to be rare, and thus it is difficult to see how random collision alone could account for so many overtly polyclonal, heterotypic tumors.

The purpose of the present article is to formalize into a statistical procedure the intuition that at low tumor multiplicity random collision should not yield a high number of heterotypic tumors. It is a slightly difficult matter to establish a useful null distribution for the number of heterotypic tumors, since it depends on unknown parameters, and requires elements from stochastic geometry to characterize collisions of random initiated cells on the two-dimensional intestinal surface. Section 2 presents distributional properties of tumor counts under random collision. These form the basis of a Bayesian analysis, described in Section 3, that generates a posterior predictive distribution for the number of heterotypic tumors by integrating over unknown parameters in the null hypothesis. The test is applied to data from Thliveris et al. (2005) and also data from Merritt et al. (1997) in Section 4.

2. Random Collision Model

In a simple version of random collision, N initiated cells appear uniformly at random over the two-dimensional surface of each mouse intestine prior to the time of observation. These initiated cells are aberrant relative to normal cells in the tissue; each such initiated cell is ancestral to a clone of descendant cells, which, by the time of observation, either forms an entire tumor or is one part of a polyclonal tumor. To analyze the random collision model, we assume that the total surface area is A square units, and that initiated cells within δ units would collide in the sense that their descendant clones would constitute a single polyclonal tumor. We take $A = 2000\,\text{mm}^2$, approximating the intestine at 400 mm in length and having uniform circumference of 5 mm. Taking $\delta = 1.5$ mm is a conservatively large estimate of the collision distance given the rather small diameters of tumors in the study by Thliveris et al. (2005).

The random placement of N initiated cells does not fully constrain the number of collisions; we may have collisions of two, three, or more initiated cells, or we may have no collisions at all. In the random graph $\mathcal{G}$ having nodes equal to the N initiated cells and having an edge between two nodes if the corresponding clones collide, each connected component is a distinct tumor. A random number x_1 of the tumors are monoclonal, each being formed from a single, isolated initiated cell. Similarly, x_2 of the tumors are biclonal, being formed from a pair of collided clones that are isolated from all others, and so on for clonality counts x_k, $k \geq 1$. Counting initiated cells, $N = x_1 + 2x_2 + 3x_3 + \cdots$. Counting tumors, we have the total tumor count in the mouse as $t = \sum_k x_k$. The probabilistic structure of $\mathcal{G}$ and the clonality counts $\{x_k\}$ is induced by the uniform random placement of initiated cells, but it represents a nontrivial stochastic process whose properties depend on N, A, and δ. With relatively small N and δ we expect very few collisions, whereas with very large N or large δ we would expect to see one large polyclonal tumor comprising numerous clones. Distribution theory is limited for the clonality counts $\{x_k\}$, though Poisson approximations can be derived using the theory of random geometric graphs (Penrose, 2003). We find quite useful an early result from Armitage (1949) that was developed in a different problem to model the counting of dust particles on a sampling plate.

Armitage derived approximations to first moments $E(x_k)$ for $k = 1, 2, 3$. They are written in terms of $\psi = \pi N \delta^2/(4A)$, which, if we view each initiated cell as the center of a disk of

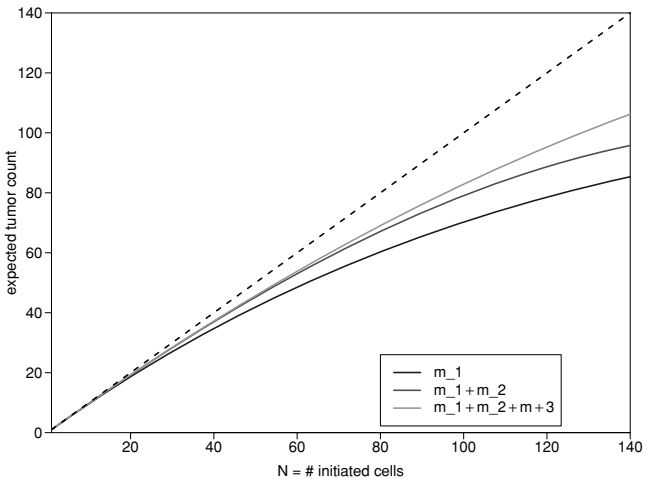

Figure 1. Expected number of tumors as a function of N for an intestinal surface of size $A = 2000\,\mathrm{mm}^2$ and a collision distance of $\delta = 1.5\,\mathrm{mm}$, based on Armitage's approximation. The expected number of tumors is less than the number of initiated cells because collision creates polyclonal tumors.

diameter δ, is the ratio of the total area of all N disks to the total area of the intestine. The approximate means are

$$\begin{aligned} m_1(N,\delta) &= N\exp(-4\psi) \\ m_2(N,\delta) &= 2N\left(\psi - \frac{4\pi+3\sqrt{3}}{\pi}\psi^2\right) \\ m_3(N,\delta) &= N\left(\frac{4(2\pi+3\sqrt{3})}{3\pi}\psi^2\right). \end{aligned} \tag{1}$$

For example, the expected number of monoclonal tumors is N times the probability that a given initiated cell forms an isolated tumor. But that requires the other $N-1$ initiated cells to be placed outside a disk of diameter 2δ centered on the given cell, which occurs with probability $(1-\pi\delta^2/A)^{N-1}$, thus leading to $m_1(N)$ above. Rather more delicate computations are used to get $m_2(N)$ and $m_3(N)$. Using elements of stochastic geometry, Armitage showed that $|m_k(N,\,\delta) - E(x_k)| = O(N\psi^3)$, and so the approximations would be useful for relatively small N and small δ (see Figure 1).

3. Posterior Predictive Analysis

To develop an inference procedure using Armitage's first-moment formulas requires a sampling model on the tumor count data. Theoretical developments might consider that the initiated cells are located at points of a homogeneous Poisson process (Hall, 1988; Penrose, 2003). In our sparse-graph setting, this leads to approximate Poisson distributions for the clonality counts $\{x_k^i\}$; the superscript i indicates the animal, and, as before, k indicates the number of collided clones. Tumor counts are well known to exhibit extra-Poisson variation, however, and so we accommodate this by introducing Gamma-distributed random effects (Newton and Hastie, 2006). Animal i is assigned the random effect z_i, which has mean 1 and variance $1/\alpha$; conditionally, on z_i and on a population-level mean parameter μ, we suppose

$$\left[x_k^i \,\middle|\, \mu, z_i, \delta\right] \sim \mathrm{Poisson}\{m_k(\mu z_i, \delta)\}. \tag{2}$$

This formulation conveniently represents the unknown, animal-specific N value (number of initiated cells) as an overall, population-level mean parameter μ adjusted by an animal-specific multiplier z_i. Alternative representations are possible, but (2) captures the main sources of variation by accommodating both polyclonality and the extra-Poisson variation of tumor counts. In this conditional Poisson model, we do not explicitly generate tumors by colliding clones, however, diagnostic calculations (see Section 4) suggest that the approximation may be reasonable. Collisions of more than three clones are not allowed in this model; such collisions seem to have very low probability in the system we have analyzed (see Figure 1). Accepting these approximations, the animal-specific tumor totals t_i have the distribution

$$[t_i \,|\, \mu, z_i, \delta] \sim \mathrm{Poisson}\{m_1(\mu z_i,\delta) + m_2(\mu z_i,\delta) + m_3(\mu z_i,\delta)\}. \tag{3}$$

Tumor size data provide some information about the collision distance δ, but it is rather indirect since any collisions leading to the polyclonal origin of a tumor may have happened much earlier than the time of observation. Our strategy is to treat δ as known in subsequent computations, fixed at some conservatively estimated value.

The first calculation uses Markov chain Monte Carlo (MCMC) to obtain a posterior distribution for unknowns μ, α, and $\{z_i\}$ given observed tumor totals $\{t_i\}$. Then we take the MCMC output and do a posterior predictive simulation of the number of heterotypic tumors in each mouse. This posterior predictive distribution is the natural reference distribution for the observed number of heterotypic tumors, and thus it generates conditional predictive p-values (Bayarri and Berger, 1999).

With n animals, say, and tumor counts $t_1,\ldots,t_n$, the posterior distribution targeted by MCMC is

$$p(z_1,\ldots,z_n,\mu,\alpha\,|\,t_1,\ldots,t_n) \propto p(\mu)p(\alpha) \times \prod_{i=1}^{n}\{p(t_i\,|\,\mu,z_i)p(z_i\,|\,\alpha)\}.$$

Here $p(z_i\,|\,\alpha)$ is the Gamma(α,α) random-effects distribution, $p(t_i\,|\,\mu,\,z_i)$ is the Poisson sampling model (3), $p(\mu)$ is a flat prior, and $p(\alpha)$ is an Exponential prior with mean 10. Some regulation of α is computationally helpful in our small sample size setting; also historical data provide some insight into typical levels of overdispersion—hence our choice for $p(\alpha)$. Ours is a routine implementation of the Metropolis–Hastings algorithm (e.g., Robert and Casella, 2002); it involves separate update steps for μ, α, and $\{z_i\}$; proposals in each step are sampled uniformly in windows centered at the current values.

The posterior distribution described above is based on tumor totals from all animals; information on how many tumors are heterotypic is not used. Our strategy is to obtain a predictive distribution for the number h_i of heterotypic tumors in animal i, and then to compare the observed heterotypic number to this distribution to obtain a conditional predictive p-value. We simulate the predictive distribution for h_i in three steps:

1. For each z_i and μ from the posterior sample, we form a trinomial probability vector

$$(p_1, p_2, p_3) = \{m_1(\mu z_i, \delta), m_2(\mu z_i, \delta), m_3(\mu z_i, \delta)\}/c,$$

where c normalizes the vector to sum to one.
2. We sample clonality counts $(x_1^i,\ x_2^i,\ x_3^i)$ as a trinomial random vector based on t_i total tumors. This step uses the well-known fact that Poisson counts become multinomials when we condition on their total. To reduce Monte Carlo error, we sample B_2 trinomial vectors for each of the B_1 posterior draws.
3. We derive heterotypic counts from the clonality counts by noting that a binomial number y_2^i of the x_2^i biclonal tumors are heterotypic and a binomial number y_3^i of the x_3^i triclonal tumors are also heterotypic. The total heterotypic count is $h_i = y_2^i + y_3^i$. Success probabilities for these binomials refer to the mechanism by which clones are assigned different types during tumor growth. The patch structure of types within tissue suggests positive association of the types of clones bound in a polyclonal tumor, however, a conservative approximation is obtained by supposing that clones are marked by independent and identically distributed Bernoulli trials (Newton, 2005). If p_i is the overall proportion of blue cells in the intestinal tissue of animal i, then the success probability for y_2^i is $2p_i(1 - p_i)$. Similarly, the success probability for y_3^i is $1 - p_i^3 - (1 - p_i)^3$.

4. Results and Discussion

Code to implement the inference calculations was developed in the R system (R Development Core Team, 2004), and was checked using several simulated data sets. We stored $B_1 = 2000$ states of the posterior sampler after subsampling from runs of length 6×10^6; output analysis indicated good mixing in the cases considered. Simulation from the predictive distribution of clonality counts used $B_2 = 1000$ conditionally multinomial draws for each of the B_1 posterior states.

Marginal posterior distributions are presented in Figure 2 for the Thliveris et al. data. Panels A and B indicate

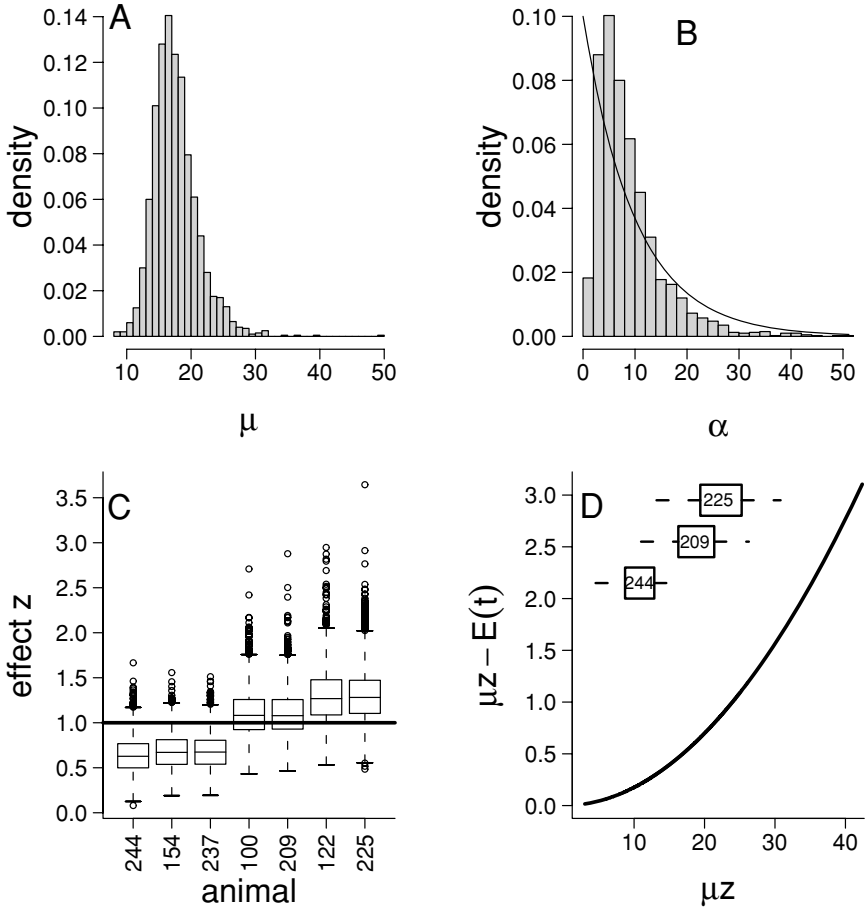

Figure 2. Posterior analysis based on $B_1 = 2000$ states subsampled from chain of length 6×10^6: Panels A and B show the marginal posterior distributions of μ and α. The Exponential prior is also drawn in panel B. Panel C shows boxplots of the posterior samples of z_i for different animals i. The prior mean of 1 unit is highlighted. The possibility for collisions is expressed in panel D by relating the animal-specific total μz to the postcollision expected count $E(t) = m_1 + m_2 + m_3$. Boxplots show the probable values of μz for three animals.

that there is much more information in the tumor counts concerning the overall mean number of initiated cells per mouse than there is about the overdispersion parameter. This finding is reasonable considering that we have only seven chimeric mice. Animal-specific posterior distributions for effects z_i reveal clear shifts from the Gamma prior in directions consistent with the tumor totals for the different animals (see Table 1). It is by virtue of the shared marginal distribution on these effects that data from the different animals are usefully combined. Otherwise animal-specific numbers of initiated cells would be unconnected and it would be difficult to bound these totals. Panel D shows that there is very little opportunity for collision given the parameter ranges active for these data. On the horizontal axis is μz, the animal-specific expected total number of initiated cells; the vertical axis compares this to the postcollision expected tumor total $E(t) = m_1 + m_2 + m_3$ as defined in model (3) and shown in Figure 1. Typically, these numbers differ by fewer than two initiated cells, indicating the vast majority of the initiated cells expected to have formed monoclonal tumors. The low rate of polyclonal tumors translates into a prediction of very few heterotypic tumors. Posterior predictive distributions for the number of heterotypic tumors in four of the seven mice are shown in Figure 3. Taken together with the relatively large observed numbers of heterotypic tumors gives small conditional predictive p-values, which are reported in Table 1 for the full set of mice. The random collision hypothesis is not a plausible explanation for the Thliveris et al. data. We applied the same procedure to tumor count data from Merritt et al. (1997). The results are more equivocal in this case (Table 2). Note that the Thliveris et al. experiment used mice with lower tumor multiplicity than those used in the 1997 study. The rationale was that a reduction in tumor number would permit a more rigorous assessment of the random collision hypothesis.

Our computations rely on a Poisson approximation to the numbers of monoclonal, biclonal, and triclonal tumors, conditional on animal-specific random effects $\{z_i\}$ (equations (1) and (2)). We checked the accuracy of this approximation by simulating random initiated clones and then counting collisions. More specifically, we simulated the posterior predictive distribution for clonality counts in a hypothetical new mouse from the Thliveris et al. population using B_1 simulated values of the mean μ and the Gamma shape α drawn from their posterior distribution (as in Figure 2). To do so we first simulated a Gamma-distributed random effect z for each (μ, α) draw. Then we simulated $N = \lfloor \mu z \rfloor$ initiated clones uniformly at random in a tubular intestinal region of length 400 mm and of circumference 5 mm. Collisions were assessed according to the spatial layout of these clones to yield the numbers of monoclonal and biclonal tumors. In parallel, we simulated Poisson-distributed monoclonal and biclonal counts using the

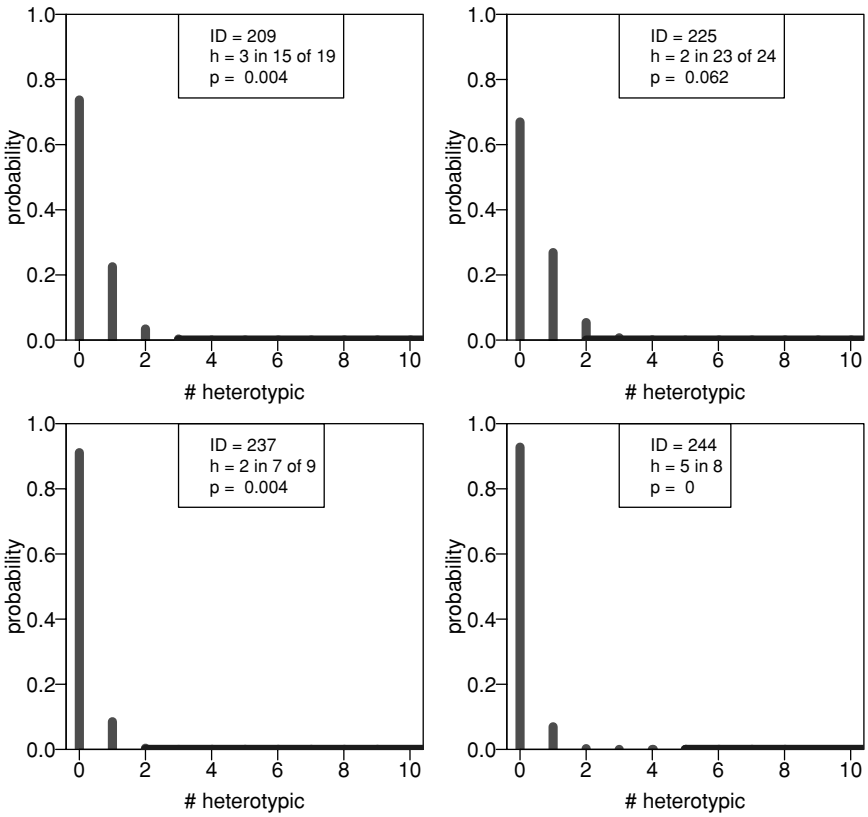

Figure 3. Posterior predictive distributions: Shown are posterior predictive distributions for the heterotypic count in four of the seven mice in the Thliveris et al. study conditional on tumor totals. Observed heterotypic counts h and p-values are indicated. For instance, 19 tumors were observed in animal 209; 15 of them gave an unambiguous phenotype, and 3 of these were heterotypic. Distributions are based on $B_2 = 1000$ simulated heterotypic counts for each of $B_1 = 2000$ posterior samples.

Table 2
Data from Merritt et al. and posterior inference: Columns as in Table 1

Mouse ID	Blue tissue (%)	Tumor count				
		Total	White	Blue	Heterotypic	p-value
112	10	105	93	5	7	0.22
113	10	155	139	1	15	0.05

Armitage rates $m_1(\mu z,\, \delta)$ and $m_2(\mu z,\, \delta)$. Figure 4 compares clonality counts derived by colliding initiated clones (left panels) to counts obtained from the conditional Poisson model (right panels). The conditional Poisson/Armitage model gives a slightly more variable predictive distribution of monoclonal counts, but there is no substantive difference between the approximation and the actual collision counts overall. Thus for the range of parameters describing the Thliveris et al. chimeric mice, the Poisson approximation to random collision is reasonable.

Some further remarks:

- Our definition of random collision entails uniform random placement of initiated cells throughout the small intestine. Nonuniform, but still random, placement would give more clustering and a higher heterotypic fraction; thus collision itself has not been rejected, just a form of completely random collision has been. Nonuniform initiation might be due to a field effect (Garcia et al., 1999). However, Thliveris et al. further considered the spatial distribution of tumors and observed that heterotypic tumors were relatively more frequent in regions of the small intestine containing fewer tumors.
- Our Bayesian approach casts the hypothesis test as a goodness-of-fit test. To do otherwise would require that we put forth some alternative hypothesis to random collision, and then to compute the probability of data on both the null and alternative hypotheses. Many alternatives (nonuniform initiation, recruitment, cooperation) would predict higher heterotypic fractions, but details of their model specifications may be hard to establish.

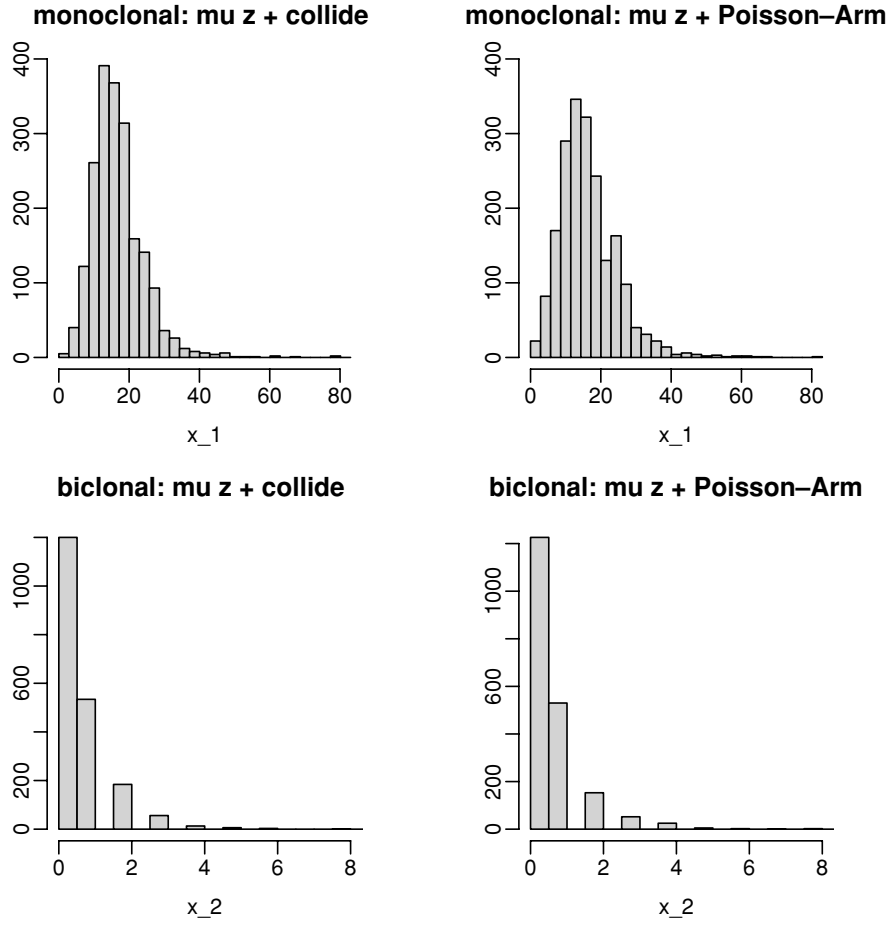

Figure 4. Comparison of Poisson/Armitage model for clonality counts (right panels) and counts obtained by colliding initiated clones (left panels). Both cases started with simulated values of $N = \lfloor \mu z \rfloor$ for the number of initiated cells. The right side then used the conditional Poisson model to generate monoclonal counts (top) and biclonal counts (bottom). Alternatively, the N initiated cells were placed uniformly at random in the intestine. The number of isolated monoclonal tumors (top) and collided pairs (bottom) were counted.

The present approach seems to be suitable for the problem at hand. Furthermore, the p-values computed are *conditional predictive* rather than *posterior predictive* because the posterior distribution driving them is based on part of the data (tumor totals) rather than all the data (tumor totals and heterotypic totals). Bayarri and Berger (1999) discuss benefits of conditional predictive p-values.

- A simple alternative hypothesis to random collision entails uniform random initiation followed by selection if initiated cells do not have enough tumorigenic potential. Such potential could be defined in terms of proximity to other initiated cells. An open statistical question is how to derive the distribution of clonality counts of higher orders under this sort of selection.

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