Evaluation of a Scenario in Which Estimates of Bioequivalence Are Biased and a Proposed Solution: $t_{\text{last}}$ (Common)

Dennis Fisher, MD\(^1\), William Kramer, PhD\(^2\), and Elise Burmeister Getz, PhD\(^3\)

Abstract

In bioequivalence (BE) testing, it is the convention to identify $t_{\text{last}}$ separately for each concentration-vs-time profile. Within-subject differences in $t_{\text{last}}$ between treatments can arise when assay sensitivity is reached during washout, causing profiles to fall below the limit of quantitation (LOQ) at different sampling times. The resulting $t_{\text{last}}$ difference may be systematic, due to true differences in exposure, and/or random, due to measurement noise. The conventional profile-specific $t_{\text{last}}$ approach assumes that concentrations in the terminal phase are sufficiently low that use of different $t_{\text{last}}$ values between treatments within a subject causes negligible bias in the AUC\(_{0-t}\) geometric mean ratio (GMR). Here we investigate the validity of this assumption. Using concentration-vs-time data following oral inhalation of 50 μg salmeterol as an example data set, we conducted simulations to evaluate whether use of different test/reference AUC timeframes arising from a systematic difference in exposure causes sufficient AUC\(_{0-t}\) GMR bias to influence the determination of BE. To ensure that results would be relevant to BE testing, we considered only test/reference relative systemic exposures within the BE window (80.00%–125.00%). We show that use of conventional profile-specific $t_{\text{last}}$ exaggerates true differences in systemic exposure; the resulting AUC\(_{0-t}\), ratios are biased from true relative exposure by an amount large enough to impact the conclusion of BE. Thus, drugs whose concentrations fall below LOQ during washout may fail BE inappropriately using conventional methods. AUC\(_{0-t}\), calculated over a common timeframe within each subject ($t_{\text{last}}$[Common]) minimizes this bias and harmonizes the statistical analysis of BE.

Keywords

AUC, bioequivalence, pharmacokinetics, salmeterol

Average bioequivalence (BE) of drugs requires that the 90% confidence interval (CI) for the geometric mean ratio (GMR) of each of 3 metrics ($C_{\text{max}}$, AUC\(_{0-t}\), and AUC\(_{0-\infty}\)) for the test product (Test) vs reference product (Reference) be fully contained within 80.00%–125.00%. It is the convention to identify the final time point for the AUC\(_{0-t}\) calculation, $t_{\text{last}}$, separately for each concentration-vs-time profile. However, if the bioanalytical assay limit of quantitation (LOQ) is reached during the washout phase, profiles for any given subject may fall below LOQ at different sampling times, in which case the conventional approach will calculate AUC\(_{0-t}\) using a different time window for Test than for Reference. In turn, AUC\(_{0-t}\) for the treatment with the earlier $t_{\text{last}}$ will be relatively underestimated (Table 1, Figure 1), thereby biasing the GMR for that metric (and, because AUC\(_{0-\infty}\) is a function of AUC\(_{0-t}\), also potentially biasing the GMR for AUC\(_{0-\infty}\)). The $t_{\text{last}}$ differences may be systematic, due to true differences in exposure, and/or random, due to measurement noise.

Recently, Allen et al.\(^1\) proposed that limiting the timeframe for calculation of AUC\(_{0-t}\) for each subject to the earliest $t_{\text{last}}$ obtained for that subject across all periods of a crossover trial, but permitting different timeframes for each subject, provides the most robust estimate of AUC\(_{0-t}\) for the purpose of a BE assessment. The inhaled fluticasone furoate BE data set reported by Allen et al. provides an extreme example of this bias because relative systemic exposures among the treatments (129% to 160%), as judged by AUC\(_{0-\infty}\), were outside the BE acceptance zone, thus causing large systematic differences in $t_{\text{last}}$ between treatments. Further, terminal AUC trapezoids each contributed substantially to the AUC\(_{0-t}\), estimate because of infrequent terminal sampling in the context of a relatively high LOQ (only 5- to 7-fold below $C_{\text{max}}$). Inclusion/exclusion of such terminal AUC trapezoids due to within-subject differences in $t_{\text{last}}$ would bias the AUC\(_{0-t}\) treatment

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\(^1\) P Less Than, San Francisco, CA, USA
\(^2\) Kramer Consulting LLC, North Potomac, MD, USA
\(^3\) Oriel Therapeutics, Berkeley, CA, USA

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Corresponding Author:
Dennis Fisher, MD, P Less Than, San Francisco, CA 94116
Email: fisher@plessthan.com
Table 1. Reference and Test Cp Profiles for the Same Subject as Figure 1†

| Time (hours) | Reference | Test |
|-------------|-----------|------|
| 0           | 0.00      | 0.00 |
| 0.05        | 106.73    | 96.057 |
| 0.067       | 111.10    | 99.990 |
| 0.083       | 103.74    | 93.366 |
| 0.117       | 86.683    | 78.0147 |
| 0.167       | 68.219    | 61.3971 |
| 0.33        | 41.215    | 37.0935 |
| 0.5         | 33.34     | 30.006 |
| 0.67        | 30.584    | 27.5256 |
| 0.83        | 29.165    | 26.2485 |
| 1.0         | 28.003    | 25.2027 |
| 1.5         | 25.141    | 22.6269 |
| 2           | 22.699    | 20.4291 |
| 4           | 15.812    | 14.2308 |
| 8           | 9.4365    | 8.49285 |
| 12          | 6.7864    | 6.10776 |
| 16          | 5.3161    | 4.78449 |
| 24          | 3.5183    | 3.16467 |
| 28†         | 2.8892    | 2.60028 |
| 32†         | 2.3756    | 2.13804 |
| 36†         | 1.9541    | 1.75869 |
| 40          | 1.6076    | 1.44684 |
| 44†         | 1.3226    | 1.19034 |
| 48          | 1.0882    | 0.97938 |
| 52†         | 0.89531   | 0.805779 |
| 56          | 0.73661   | 0.662949 |

†Test values were obtained by multiplying Reference values by a factor (0.90 in this example). Boldfaced entries are samples <LOQ (1.00 pg/mL) that were excluded from calculations of AUC0-t and half-life. At 48 hours (shaded row), the samples straddled the LOQ; thus, this subject was “unmatched.”

Because blood concentrations during washout are typically low relative to Cmax, the conventional assumption is that bias caused by use of different tlast values across treatments within a subject is negligible, especially if true relative exposure between treatments is not substantially different from unity. Here, we investigate the validity of this assumption using an example drug, salmeterol administered by oral inhalation. To generate results relevant to most BE determinations, we simulated only situations in which true Test-vs-Reference differences in systemic exposure are within the BE requirement (80.00%–125.00%), and terminal AUC trapezoids are a small percentage of total AUC.

We extend the analysis to consider the magnitude of error introduced to AUC0-t. Finally, we explore the sensitivity of our results to the blood-sampling schedule.

Methods

Pharmacokinetic data from a clinical trial were obtained from the Novartis clinical trials library. Salmeterol (50 μg) was administered on 4 occasions to 60 healthy adult male and female subjects as a single dose by oral inhalation. The interval between treatments was

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Figure 1. Hypothetical plasma concentration (Cp) profiles for Reference (left) and Test (right) products are displayed on semi-log coordinates. Both profiles are based on predicted values for 1 subject; values for Test are exactly 90% of the values for Reference. A thin horizontal line appears in both panels at Cmax of Reference. With an LOQ of 1.00 pg/mL (dashed line), blue circles represent “observed” samples and X indicates samples reported as <LOQ. AUC0-t calculations for Reference include entire area through 48 hours, whereas calculations for Test are truncated at 40 hours. As a result, the pink shaded region (hours 40–48) is included in the calculations of AUC0-t for Reference but not for Test. Half-life (calculated from linear regression of the log of the final 3 samples >LOQ) was slightly longer for Reference than Test (r for the linear regression is displayed). The fit of the linear regression (extrapolated through hour 56) is shown as a thick red line.
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glądays. Plasma was sampled at frequent intervals for 56 hours for each session; after 24 hours, the interval was 8 hours (24, 32, 40, 48, and 56 hours). Plasma concentration (Cp) was determined using a validated high-performance liquid chromatography/tandem mass spectrometry assay with an LOQ of 1.00 pg/mL. A data set suitable for a mixed-effects population analysis with NONMEM (Version 7.3.0, ICON Development Solutions, Hanover, Maryland) was assembled using R (Version 3.2.0, www.R-project.org). The data set included the first session for each subject. Actual sample times were used in the NONMEM analysis; predictions were obtained at all nominal times (and at 4 additional timepoints: 28, 36, 44, and 52 hours). Linear 2- or 3-compartment models with first-order absorption from a depot were fit to the data.

To determine the impact of true relative bioavailability deviating from unity on calculations of GMR and 90% CI for each of AUC_0-t and AUC_0-\infty, we performed the following steps for bioavailability factors (F) ranging from 80% to 95% in increments of 1%:

1. For each subject, the predicted Cp profile (excluding measurement error; through the sample at 56 hours) was reviewed; concentrations < LOQ were discarded. These concentrations, the corresponding sample times, and subject IDs were included in the “Reference” data set. Next, each Cp value was multiplied by F. The adjusted Cp values > LOQ, corresponding sample times, and subject IDs were included in the “Test” data set. For each subject, 2 scenarios existed. In one, in which the subject was identified as “unmatched,” multiplying Reference concentrations by F caused 1 or more Test Cp values to become < LOQ (eg, if a Reference sample had a value of 1.02 pg/mL, F = 0.9 would cause the corresponding Test sample to have Cp less than the LOQ of 1.00 pg/mL). If none of the Test Cp values was < LOQ, the subject was identified as “matched” to indicate that the timeframe over which there was quantifiable data was identical for Test and Reference. Data from a representative subject are shown in Table 1.

2. For each subject, for each of the Reference and Test profiles, AUC_0-t (using the linear trapezoidal method), terminal rate constant (based on the final 3 time points), AUC_tail, and AUC_0-\infty (the sum of AUC_tail and AUC_0-t) were calculated using standard methods. If a subject was matched, the same time points were used in the linear regression; in turn, terminal rate constant and terminal half-life were identical for Test and Reference. Because linear regressions were based on predicted Cp (without residual error), r^2 for the linear regression was always >0.95. Median values for AUC_tail (across F values) were typically 11% of AUC_0-\infty.

3. For each of AUC_0-t and AUC_0-\infty, GMR and 90% CI were calculated based on log-transformed values using a standard ANOVA model. Because data were simulated, period and sequence were not used as terms in the ANOVA model. Three sets of analyses were performed:

a. All subjects
b. Only matched subjects
c. Only unmatched subjects

4. Analyses were performed with and without the additional sampling times (28, 36, 44, and 52 hours) to examine whether the findings were robust with a different sampling schedule.

5. Graphics were prepared to show:

a. Estimated relative F vs the true value used in the simulation.
b. Lower bounds of the 90% CI for each of AUC_0-t and AUC_0-\infty vs the true value used in the simulation.

All calculations (except the initial fitting performed with NONMEM) were conducted using R.

**Results**

**Analyses Based on the Nominal Sampling Regimen**

GMR for both metrics were further from unity relative to the true value (Table 2, Figure 2); the magnitude of the difference between estimated and true

| True Value (%) | AUC_0-t | AUC_0-\infty |
|----------------|---------|-------------|
|                | All     | Unmatched   | All     | Unmatched   |
| 80             | 76.70   | 74.27       | 79.65   | 79.38       |
| 81             | 78.00   | 75.30       | 80.67   | 80.37       |
| 82             | 79.32   | 76.35       | 81.72   | 81.40       |
| 83             | 80.61   | 77.59       | 82.74   | 82.41       |
| 84             | 81.75   | 78.49       | 83.75   | 83.37       |
| 85             | 82.84   | 79.47       | 84.75   | 84.34       |
| 86             | 83.95   | 80.53       | 85.75   | 85.32       |
| 87             | 85.00   | 81.41       | 86.76   | 86.31       |
| 88             | 86.13   | 82.24       | 87.76   | 87.24       |
| 89             | 87.20   | 83.14       | 88.76   | 88.21       |
| 90             | 88.35   | 84.30       | 89.83   | 89.39       |
| 91             | 89.33   | 85.24       | 90.82   | 90.38       |
| 92             | 90.51   | 86.18       | 91.86   | 91.45       |
| 93             | 91.67   | 87.02       | 92.87   | 92.41       |
| 94             | 93.18   | 88.64       | 93.93   | 93.50       |
| 95             | 94.26   | 89.61       | 94.93   | 94.50       |

aGMR for AUC_0-t and AUC_0-\infty for all subjects and unmatched subjects as a function of relative F. For matched subjects, GMR was identical to the true value in all instances.
AUC ratios was always larger for AUC_{0-t} than for AUC_{0-\infty}. In subset analyses for unmatched subjects, deviations for both metrics were larger compared to the analysis that pooled matched and unmatched subjects (“all subjects”). In contrast, in subset analyses for matched subjects, GMR for both metrics matched the true value. The CIs for these metrics, although narrow (a result of both the absence of within-subject variability and the identical true relative exposure value applied to every subject with these simulated data), reflect the larger bias from true relative bioavailability for AUC_{0-t} relative to AUC_{0-\infty}. With a true relative bioavailability of 82%, the lower bound of the confidence interval for all subjects was 78.61% for AUC_{0-t} and 81.58% for AUC_{0-\infty}.

Table 3. Results (Expressed as %) for Unmatched Subjects and All Subjects From the Analysis Based on the Nominal Sampling Regimen\(^6\)

| Population   | N   | AUC_{0-t} GMR | Lower Bound | Upper Bound | AUC_{0-\infty} GMR | Lower Bound | Upper Bound |
|--------------|-----|---------------|-------------|-------------|---------------------|-------------|-------------|
| All          | 60  | 79.32         | 78.61       | 80.02       | 81.72               | 81.58       | 81.86       |
| Unmatched    | 28  | 76.35         | 75.62       | 77.09       | 81.40               | 81.12       | 81.67       |

\(^6\)With a relative F of 0.82 (82%) and LOQ = 1.00 pg/mL. For matched subjects (N = 32), all individual T/R ratios were identical to the true value (82.00%); therefore, the GMR was identical to the true value, and the confidence interval had zero width. The lower bounds for AUC_{0-t} and AUC_{0-\infty} for all subjects (boldfaced) straddled the 80.00% acceptance value.

Figure 2. Values for GMR for AUC_{0-t} (left) and AUC_{0-\infty} (right) are displayed against true relative bioavailability (top, nominal sampling regimen; bottom, supplemented sampling regimen). Colors distinguish all, unmatched, and matched subjects; the line of identity is displayed in black.
The difference between the lower confidence bounds for AUC\textsubscript{0,\infty} relative to AUC\textsubscript{0,t} in the “all subjects” population increased as the true relative bioavailability value deviated further from unity. This same situation applied for the subset analysis for unmatched subjects (not shown). However, consistent with estimates for matched subjects matching the true value (and with no intrasubject variability), the lower and upper bounds of the confidence interval were identical to the GMR in the “matched subjects” population (not shown).

**Analyses Based on the Supplemented Sampling Regimen**

Denser sampling during late washout increased the number of unmatched subjects (compare counts in the panels in Figure 3). However, deviations of GMR from true relative F for both metrics for unmatched subjects were slightly smaller compared to the nominal sampling regimen (Table S1, Table S2). Thus, because of the balance between the greater number of unmatched subjects and the smaller bias among these unmatched subjects, there was little change in the magnitude of GMR bias in the “all subjects” population with the denser sampling regimen. Lower confidence bounds were similar to those from the nominal sampling regimen. Two pairs of lower bounds straddled the 80.00% value.

**Discussion**

We demonstrate that the magnitude of bias incurred by use of different t\textsubscript{last} values between treatments within a subject for AUC\textsubscript{0,4} calculation is not negligible and might lead to an incorrect conclusion with respect to bioequivalence. If systemic drug concentrations fall below LOQ during the washout phase, AUC\textsubscript{0,4} GMR estimates of relative F exaggerate true deviations of relative F from unity. In turn, BE analyses for drugs with terminal phase concentrations near assay sensitivity may fail inappropriately. Separate evaluations of matched vs unmatched subjects identify the source of the problem: as illustrated in Figure 1, the problem with AUC\textsubscript{0,4} results from its calculation over different time windows when there is systematic truncation of Cp data < LOQ at different t\textsubscript{last} values. When t\textsubscript{last} is matched within a subject [ie, using the earliest t\textsubscript{last} obtained across all periods of the crossover trial, t\textsubscript{last}(common)], there is no longer a significant bias in the GMR (or confidence interval) for AUC\textsubscript{0,4}.

We agree with the Allen et al proposal that when blood/plasma concentrations fall below LOQ during the terminal phase, calculation of AUC\textsubscript{0,4} to assess bioequivalence should use a single t\textsubscript{last}(common) for each subject instead of the t\textsubscript{last} of each individual profile. This approach should yield consistent results across pharmacokinetic BE studies, minimizing the opportunity for the BE conclusions to depend on the interaction among LOQ, sampling schedule, and active ingredient kinetics. This is particularly relevant for products for which systemic exposure is intended to be low (and, therefore, terminal phase concentrations fall below LOQ) such as orally inhaled salmeterol, used as an example here, or orally inhaled fluticasone furoate, the example offered by Allen et al, or for products with higher systemic exposure but for which current technology cannot improve the assay LOQ. We propose that the AUC BE requirement should be harmonized across all BE studies and that the t\textsubscript{last}(common) approach, as presented here, meets that objective.

Although the opportunity for bias from the true exposure ratio when different time windows are used in the AUC\textsubscript{0,4} calculation may be self-evident, Allen
et al provided no assessment of whether the magnitude of this bias could account for the observed difference in GMR between AUC_{0-t} and AUC_{(0-t)\text{common}}, nor did they assess the impact of this bias on a bioequivalence interpretation. We present a quantitative assessment of the magnitude of the bias, identifying the root cause—different time windows in the AUC calculations—and demonstrating that the magnitude of this bias can be sufficiently large to affect the bioequivalence outcome, always exaggerating the true exposure difference between Test and Reference.

One implication of truncating the Cp profile at t_{last}(\text{common}), compared to t_{last}, is that data are removed from the analysis, generally an anathema to regulatory bodies. However, the data that would be removed from the analysis under this approach are those closest to the assay LOQ, for which variability is often largest. Further, AUC_{0-\infty} provides a logical companion metric to AUC_{(0-t)\text{common}} in that a common time window for the AUC calculation is achieved by each of truncation (AUC_{(0-t)\text{common}}) and extrapolation (AUC_{0-\infty}).

Our analyses show a sharp distinction between subjects designated as matched vs unmatched. For subjects designated as matched, GMR is identical to the true value. In contrast, for subjects designated as unmatched, GMR and lower bounds of the CI deviate from the true value; however, the magnitude of deviation is markedly larger for AUC_{0-t} compared to AUC_{0-\infty} because the mismatch in the time window in the AUC_{0-t} calculation is counterbalanced by the extrapolation to infinite time in AUC_{0-\infty}. In turn, if the number of unmatched subjects is large, AUC_{0-t} may be sufficiently biased relative to the true value to cause a BE study to fail erroneously. In contrast, the error in AUC_{0-\infty} is relatively small and less likely to cause a BE study to fail.

The critical element in our calculations is the AUC in the pink region in Figure 1. Analyses in which we simulated additional samples during washout examined 2 issues:

1. Denser sampling might decrease the width of that pink region.
2. Some subjects previously designated as matched might become unmatched.

On balance, adding samples during washout had minimal impact on the deviations between the lower bounds of the 2 metrics.

Having established that the conventional AUC_{0-t} calculation leads to a biased GMR estimate, we note that the GMR and CI for both metrics were identical to the true values for matched subjects. This supports our claim that calculating both metrics over the same time interval mitigates biases introduced when AUC_{0-t} is calculated over different timeframes for each of 2 treatments. Although removing data from the analysis can introduce bias to the metrics themselves, in this instance the imbalance in data in unmatched subjects creates a bias in the treatment ratio that is large enough to alter a BE determination. Had we applied a common t_{last} [which we term t_{last}(\text{common})] to each subject, the estimates of AUC_{0-t} would each be biased, but to the same extent; in turn, their ratio (the value of interest in a BE study) would be unbiased under the circumstances of the present analysis. In a real-world situation that includes both measurement error and within-subject variability in both relative F and the shape of the concentration profile, factors in addition to differences in timeframe also influence calculation of the AUC. In these circumstances, the t_{last}(\text{common}) approach mitigates bias introduced by differences in timeframe and should be considered an appropriate approach.

Two alternative approaches might minimize the opportunity for subjects to be mismatched, thus minimizing the bias between true and estimated AUC GMRs. The first involves truncating sampling at a time when all samples are likely to be > LOQ. Although this might assure that all subjects are matched, it might provide biased estimates for the AUC metrics if absorption and distribution are incomplete. The second approach is to administer a higher-than-marketed dose, yielding higher Cp values and increasing the duration over which the Cp profile is > LOQ. If this decreased the number of unmatched subjects, it might eliminate the problem that Allen et al identified. However, accepting the results of such an analysis requires the assumption that data from a higher-than-marketed dose are relevant to the marketed dose.

Several issues of our analysis plan warrant comment. First, we used predicted values obtained from a compartmental model (and obtained predictions at nominal times to ensure that each subject’s sample times were identical). Use of predicted values eliminated noise prevalent with observed concentrations; this assured that assay and/or physiologic variability did not impact the last quantifiable value or estimation of terminal half-life. The linear regression used for calculation of the terminal rate constant (and, in turn, AUC_{tail} and AUC_{0-\infty}) yielded coefficients of determination close to unity (with the nominal sampling regimen, r^2 was always ≥ 0.97). In addition, this approach assured that the AUC_{tail} was a small fraction of AUC_{0-\infty} (with the nominal sampling regimen, median values were typically 11%).

We calculated the Test Cp profile by multiplying the Reference Cp profile by a factor. This resulted in the 2 profiles having identical shapes (particularly evident in
graphics in the log domain). This approach minimized intrasubject variability, as evidenced by the complete lack of intrasubject variability in matched subjects. In turn, the confidence intervals were unrealistically small, their magnitude depending on the fraction of unmatched vs matched subjects. One consequence of these artificially small CIs is that the GMR associated with the CI for the 2 AUC metrics straddling the 80.00% BE criteria was close to 80.00%. With “real” data, it is possible that a GMR close to, or even exceeding, 90% could be associated with CIs for the 2 AUC metrics that straddle 80.00%. Had our analyses incorporated more within-subject variability, we could have increased the width of the CI to that typically seen with experimental data. Regardless, the present approach demonstrates the impact on both the GMR and CI for each metric.

Finally, we present results for scenarios with relative $F < 100\%$. Additional simulations (not reported) confirm a similar deviation with relative $F > 100\%$.* This is expected because BE analyses (which are conducted in the log domain) are symmetric with respect to 100% in the log domain (eg, relative F of 90.00% for Test vs Reference is identical to relative F of 111.11% for Reference vs Test).

Several investigators have examined the impact of duration of sampling on noncompartmental estimation of AUC$_{0-t}$. El-Tahtawy et al$^2$ concluded that sampling should not extend excessively. Colucci et al$^3$ concluded that the sampling regimen should cover at least 2-4 half-lives. However, neither of these investigators examined the discrepancy between AUC$_{0-1}$ and AUC$_{0-\infty}$.

Our findings support the decision of Allen et al to apply a $t_{\text{last}}$(common) approach for the calculation of AUC$_{0-t}$ geometric mean ratios and confirm that this approach is both necessary and sufficient to address the otherwise inherent bias in the AUC$_{0-t}$ GMR for drugs that fall below LOQ during washout. The $t_{\text{last}}$(common) approach serves to harmonize the metrics used for the statistical analysis of pharmacokinetic BE across all BE studies. It remains to be seen if regulatory bodies will consider this approach.

**Declaration of Conflicting Interests**
D.F. and W.K. are paid consultants to Oriel Therapeutics. E.B.G. is an employee of Oriel Therapeutics.

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**Supporting Information**
Additional Supporting Information may be found in the online version of this article at the publisher’s website.

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*This requires that the Reference values for at least 1 subject reach LOQ before the 56-hour sample. If the 56-hour Reference samples for all subjects were >LOQ and F is >100%, all subjects would be matched, and timeframe would be identical for all subjects.