Cancer Immunology Miniatures

Cancer Immunology Research

JAK2 Expression Is Associated with Tumor-Infiltrating Lymphocytes and Improved Breast Cancer Outcomes: Implications for Evaluating JAK2 Inhibitors

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Abstract

Janus kinase-2 (JAK2) supports breast cancer growth, and clinical trials testing JAK2 inhibitors are under way. In addition to the tumor epithelium, *JAK2* is also expressed in other tissues including immune cells; whether the *JAK2* mRNA levels in breast tumors correlate with outcomes has not been evaluated. Using a case–control design, *JAK2* mRNA was measured in 223 archived breast tumors and associations with distant recurrence were evaluated by logistic regression. The frequency of correct pairwise comparisons of patient rankings based on *JAK2* levels versus survival outcomes, the concordance index (CI), was evaluated using data from 2,460 patients in three cohorts. In the case–control study, increased *JAK2* was associated with a decreasing risk of recurrence (multivariate $P = 0.003$, $n = 223$). Similarly, *JAK2* was associated with a protective CI (<0.5) in the public cohorts: NETHERLANDS CI = 0.376, $n = 295$; METABRIC CI = 0.462, $n = 1{,}981$; OSLOVAL CI = 0.452, $n = 184$. Furthermore, *JAK2* was strongly correlated with the favorable prognosis *LYM* metagene signature for infiltrating T cells ($r = 0.5$; $P < 2 \times 10^{-16}$; $n = 1{,}981$) and with severe lymphocyte infiltration ($P = 0.00003$, $n = 156$). Moreover, the JAK1/2 inhibitor ruxolitinib potently inhibited the anti-CD3–dependent production of IFN-γ, a marker of the differentiation of Th cells along the tumor-inhibitory Th1 pathway. The potential for JAK2 inhibitors to interfere with the antitumor capacities of T cells should be evaluated. *Cancer Immunol Res; 2(4); 301–6. ©2014 AACR.*

Introduction

Janus kinase-2 (JAK2) is essential for the signaling of a variety of cytokine receptors, including receptors for erythropoietin during erythropoiesis and for prolactin during mammary differentiation (1, 2). JAK2 has emerged as an important target in myeloproliferative disorders, and increasingly, in solid tumors such as breast cancer. Recent studies have implicated JAK2 in interleukin (IL)-6–dependent breast cancer stem cell self-renewal (3), and in both IL-6- and IL-8–dependent growth of triple-negative breast cancers (4). Furthermore, JAK2 signaling has been implicated as a mechanism of escape from other targeted breast cancer therapies (5). Thus, JAK2 inhibitors are being evaluated in patients with breast cancer (6). JAK2 is also expressed in diverse cell types, including immune cells, and whether the overall *JAK2* mRNA levels in breast tumors are associated with clinical outcomes has not been evaluated.

Studies of mRNA levels in primary breast tumors have been useful for classifying breast cancers into subtypes that correlate with prognosis and drug responsiveness (7–9), for predicting recurrence (10, 11), and for delineating gene expression signatures that correlate with prognosis (12–15). Here, we evaluated the association between tumor mRNA levels of *JAK2* and clinical outcomes in a novel case–control study and in three public cohorts. Outcomes included distant metastatic recurrence in a matched case–control study ($n = 223$); recurrence-free survival in the Netherlands Cancer Institute cohort that was used to develop the MammaPrint recurrence risk test ($n = 295$; ref. 11); overall and disease-specific survival in METABRIC, currently the largest collection of gene expression and copy-number data linked to long-term breast cancer outcomes ($n = 1{,}981$; ref. 14); and overall survival in OSLOVAL, a recent cohort that, along with METABRIC, formed the basis of the Sage Bionetworks DREAM breast cancer prognosis challenge ($n = 184$; refs. 12, 15).

Materials and Methods

Selection of cases and controls

The protocol to use a breast cancer research database for case selection, to access institution-archived leftover tumor tissue, and to undertake molecular biology studies of the tissue was approved by the Institutional Review Boards of the Fred Hutchinson Cancer Research Center (File 6643) and the

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Swedish Medical Center (File 4924C-10). Patient consent was not required. The breast cancer research database at the Swedish Cancer Institute contains patient, tumor, treatment, and outcomes data collected since 1989 for more than 12,000 patients. The dataset was reduced to women followed for at least 2 years with invasive carcinoma with T1–3 primary tumors and treated by partial mastectomy plus breast irradiation or total mastectomy, sentinel node biopsy or axillary dissection, and adjuvant chemotherapy. Patients with multiple primaries, T4 primaries or distant metastases, and those receiving neoadjuvant chemotherapy, were excluded. Matching variables included extranodal extension of metastasis, lymphovascular invasion, estrogen receptor (ER)/progesterone receptor (PR)/human epidermal growth factor receptor-2 (HER2) status, T-stage, and N-stage. A T–N interaction term allowed for the fact that tumor size is more important for women without positive nodes than for women with positive nodes. Within each matched pair, diagnosis dates of the recurring and nonrecurring patients were no more than 2 years apart. Propensity scoring was used to match 112 cases of distant recurrence following surgery to 112 nonrecurring controls using the "Optmatch" package (16) and R (17).

Quantitative Reverse Transcriptase PCR

RNA was extracted from 4 × 10 μm sections using the Absolutely RNA FFPE System (Stratagene). The amount of tumor versus normal tissue in each section was greater than 50% for 84% of samples and greater than 90% for 47% of samples as determined by pathologists' inspection of hematoxylin and eosin–stained slides. cDNA was synthesized using random hexamers and SuperScript III (Invitrogen) and was preamplified for 14 cycles using the Taqman preamplification system (Applied Biosystems). All probes bound exon junctions to prevent genomic DNA amplification (Supplementary Table S1). Diluted cDNA was used to seed triplicate real-time PCR reactions for each Taqman assay using standard cycling conditions. Cycle threshold (C_t) values were determined using Sequence Detection Software (Applied Biosystems). Relative quantification was calculated as $2^{-\Delta C_t}$, where ΔC_t values were calculated by subtracting the indicated control gene mean C_t value from the target gene mean C_t value.

JAK2 mRNA levels and distant recurrence in the case–control study

Associations between *JAK2* mRNA and the likelihood of recurrence were evaluated by logistic regression. In the continuous model, coefficients were calculated as estimates of the change in the log of the odds that an individual experienced a recurrence for every 2-fold increase in *JAK2* levels, in which a negative coefficient indicates that increasing *JAK2* levels are associated with a decreasing likelihood of recurrence. Because the participants had one ($n = 184$), two ($n = 26$), or three tumor specimens ($n = 14$), generalized estimating equations were used to account for varying numbers of tissues per individual ("Geepack" package; ref. 18). Regressions were also performed using only primary tumors. For this, among the 26 individuals with both a primary and node specimen, only the primary tissue was included, and data from 31 individuals with only a node specimen were excluded. In the dichotomous model, coefficients were calculated with above-median versus below-median *JAK2* levels as a predictor of recurrence, in which multiple specimens were averaged to one value per individual. Multivariate analysis adjusted for clinical factors with which *JAK2* expression was significantly correlated.

JAK2 mRNA levels and survival outcomes in the public cohorts

The inclusion criteria, clinical characteristics, and follow-up of the NETHERLANDS, METABRIC, and OSLOVAL cohorts were described (11, 12, 14). Data are available via Sage Bionetworks (www.synapse.org) under the following identifiers: doi:10.7303/syn4517.1, doi:10.7303/syn1688369, and doi:10.7303/syn1688370. Concordance index (CI) values were determined as described (12, 19).

Results

Case–control study of *JAK2* mRNA levels and distant recurrence

We previously optimized methods for measuring *JAK2* mRNA in formalin-fixed, paraffin-embedded tumors by quantitative reverse transcriptase PCR (qRT-PCR) despite the degradation that characterizes RNA extracted from these samples (20). We applied this approach to tumor specimens from 112 women who underwent surgery for breast cancer and subsequently experienced a distant metastatic recurrence and 112 women who did not. With the exception of a borderline significant increase in the number of ER^- tumors among recurrences, there were no significant differences in clinical characteristics between cases and controls (Supplementary Table S2). Of note, although ER was a matching variable, it was not the only variable, which accounts for the residual effect of this strong prognostic factor even after propensity score matching. Sufficient RNA was available in 223 tumor specimens for *JAK2* mRNA determinations. The validity of our mRNA measurements was confirmed by (i) the strong correlation in values obtained using probes for both *JAK2 exon8/9* and *exon23/24*; (ii) the reproducible mRNA levels across three separate tumor specimens for 14 tumors for which this comparison was possible; and (iii) the strong concordance in mRNA levels of *ESR1*, *PGR*, *ERBB2*, and the corresponding clinical immunohistochemistry results (Supplementary Fig. S1).

JAK2 mRNA levels were significantly higher in tumors from women who experienced no distant recurrence compared with those who experienced a distant recurrence (Fig. 1A). This association was significant for both *JAK2 exon8/9* and *exon23/24* probes in logistic regression when *JAK2* mRNA was treated as a continuous or dichotomous variable (Supplementary Table S3). Furthermore, with the exception of the *JAK2 exon8/9* probe in the dichotomous model, significance was maintained in multivariate analysis. The association between increasing *JAK2* mRNA and decreasing distant recurrence was also significant when analysis was restricted to primary tumors. A receiver operator curve revealed that the association between higher *JAK2* mRNA and reduced recurrence was maximal when tumor samples with the top 40% to 50% of *JAK2* expression level were defined as high *JAK2* (Fig. 1B).

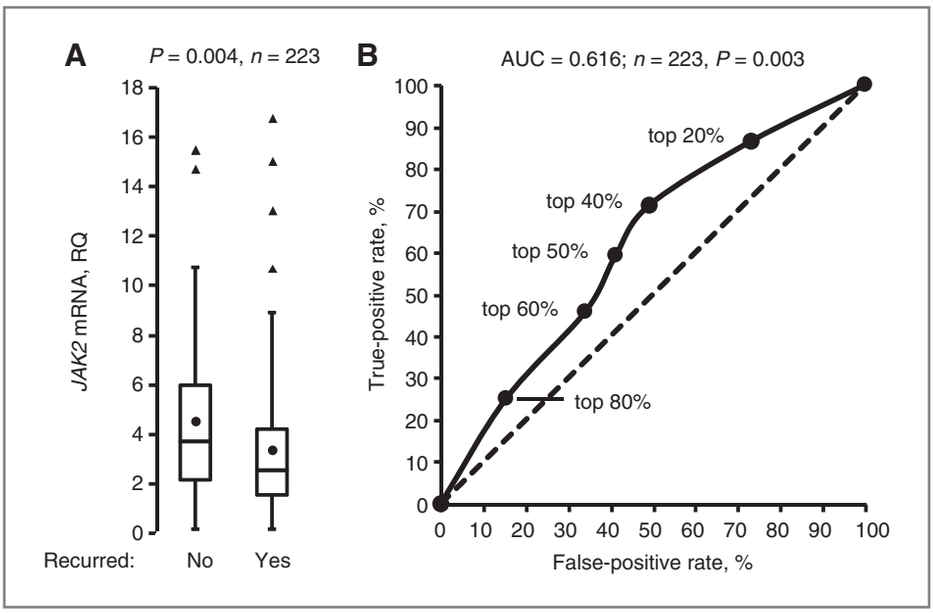

Figure 1. *JAK2* mRNA is associated with reduced distant breast cancer recurrence. A, *JAK2* mRNA was measured by qRT-PCR using RNAs extracted from 223 breast tumor samples. Values for the *JAK2 exon23/24* junction probe are shown normalized to the endogenous control gene *HMBS*. Box plots depict the distribution of normalized *JAK2* mRNA values in quartiles. Circle in the box, mean value; horizontal line, median value; triangles, outliers. The *P* value was calculated using the *t* test. RQ, relative quantification. B, *JAK2* mRNA was modeled in a receiver operator curve as a predictor of reduced recurrence across different thresholds for defining high *JAK2*. The true-positive rate versus false-positive rate of distant recurrence for each model versus actual outcomes is plotted for each threshold of defining high *JAK2*. The percentage of samples that are defined as high *JAK2* is shown for each model (top %). The *P* value for the area under the curve (AUC) was calculated using the *Z* test.

JAK2 mRNA levels and survival outcomes in the public cohorts

Next, we evaluated the association between *JAK2* mRNA and outcomes in the NETHERLANDS, METABRIC, and OSLOVAL cohorts. We used the CI (19), which provides a convenient measure of the strength and direction of an association between a single gene and outcomes, to score submissions in the Sage Bionetworks DREAM breast cancer prognosis challenge (12). The CI is the relative frequency of correct pairwise comparisons of patient rankings based on gene expression levels versus survival outcomes. A CI > 0.5 indicates that higher expression is associated with shorter survival, whereas a value of <0.5 indicates that higher expression is associated with longer survival. For example, using disease-specific survival data in METABRIC, the single-gene mRNA with the poorest prognosis was previously found to be *CDCA5* with a CI of 0.651, indicating that if 2 patients were randomly selected, the patient with the higher *CDCA5* level will have shorter survival 65.1% of the time (12). Conversely, the single most protective gene was *FGD3* with a CI of 0.352, indicating that if 2 patients were randomly selected, the patient with the higher *FGD3* level will have the longer survival 64.8% (100%–35.2%) of the time.

JAK2 mRNA exhibited a protective CI in all three datasets (Fig. 2). The strongest effect was observed in the NETHERLANDS cohort, in which the CI of 0.376 indicates that if 2 patients were randomly selected, the patient with the higher tumor *JAK2* mRNA level would have the longer recurrence-free survival 62.4% (100%–37.6%) of the time. Similarly, *JAK2* mRNA was consistently protective, albeit to a lesser extent, in the METABRIC and OSLOVAL cohorts. Because METABRIC provided a sufficient sample size, we also evaluated the CI for *JAK2*

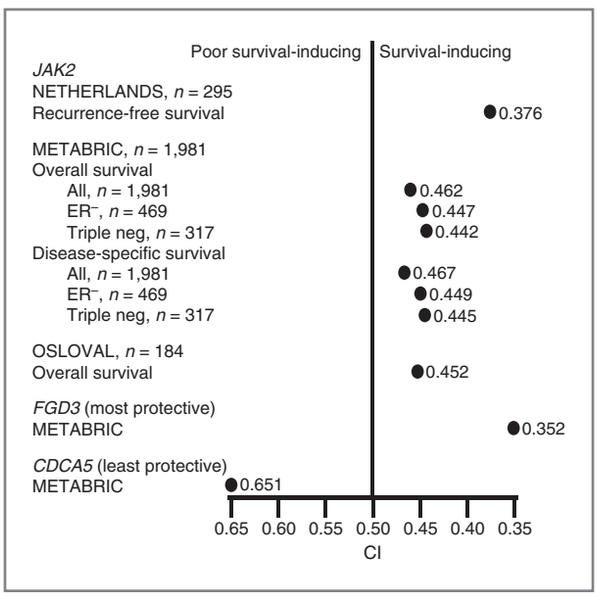

Figure 2. *JAK2* mRNA is associated with a protective CI in the NETHERLANDS, OSLOVAL, and METABRIC cohorts. The CI in patient rankings based on *JAK2* mRNA versus survival outcomes is shown for each indicated cohort. For comparison, the CIs for the least and most protective single genes in METABRIC (*CDCA5* and *FDG3*) are shown.

mRNA in ER$^-$ and ER$^-$/PR$^-$/HER2$^-$ (triple-negative) subtypes; *JAK2* mRNA was even more protective for both overall and disease-specific survival in these subtypes.

JAK2 mRNA and protein levels

To explore the mechanism by which *JAK2* mRNA is associated with favorable prognosis, we investigated the relationship between *JAK2* mRNA and protein levels. The specificity of a total JAK2 antibody was validated by the strong correlation between *JAK2* mRNA and protein levels (as measured by Western blotting) in a panel of 18 human breast cancer cell lines and by the ability to discriminate between *JAK2*-deficient γ2A cells (21) and *JAK2*-transfected γ2A cells in immunohistochemistry (Supplementary Figs. S2 and S3). We then measured 10 tumors from the case–control study randomly selected from the highest quartile of *JAK2* mRNA expression and 10 tumors from the lowest quartile. Immunohistochemical staining ranged from absent (0) to robust (3+) and was prominent in the tumor epithelial cells. However, JAK2 antibody staining levels in the tumor epithelial cells were not correlated with overall tumor *JAK2* mRNA levels ($r = -0.17$, $n = 20$; Supplementary Table S4). This finding suggests that the association between higher *JAK2* mRNA levels and favorable outcomes in breast cancer may not be a result of JAK2 protein function in breast tumor epithelial cells, and that other cell types in the primary tumors that are associated with prognosis contribute to overall *JAK2* mRNA levels.

JAK2 mRNA levels and tumor-infiltrating T cells

JAK2 is expressed in immune cells, and tumor-infiltrating lymphocytes, especially T cells, have been associated with favorable breast cancer prognosis (14, 22). We, therefore, tested whether breast tumor *JAK2* mRNA levels correlate with the T-cell transcript-enriched *LYM* metagene signature. The *LYM* metagene is associated with favorable prognosis in breast cancer, in particular in ER$^-$ breast cancer and even more so in the absence of multiple positive lymph nodes, and recently formed part of the winning prognostic model in the Sage Bionetworks DREAM breast cancer prognosis challenge (12, 15). The *LYM* metagene was recently defined with increased accuracy, following mining from data sets from multiple cancer types available from The Cancer Genome Atlas (23). Indeed, there was a highly significant correlation between *JAK2* mRNA levels and the *LYM* metagene in tumor samples from METABRIC (Fig. 3A). In contrast, the *LYM* metagene had an inverse correlation with the breast epithelial-associated transcript *ESR1* (Fig. 3B). Furthermore, *JAK2* mRNA levels were correlated strongly with levels of infiltrating lymphocytes as determined by pathologic assessment in a subset of 156 tumors for which these data were available (Fig. 4). These tumor samples belonged to METABRIC integrative cluster 4, which was previously associated with favorable prognosis and a strong adaptive immune response signature (14). Finally, consistent with a functional role for JAK2 in supporting cytokine receptor signaling during T-cell activation, we found that the JAK1/2 inhibitor ruxolitinib markedly inhibited the anti-CD3–dependent production of IFN-γ, a marker of the differentiation of Th cells along the tumor-inhibitory Th1 pathway (Supplementary Fig. S4; 24). These results suggest that the consistently protective effect of *JAK2* mRNA is related, at least in part, to the levels of infiltrating T cells.

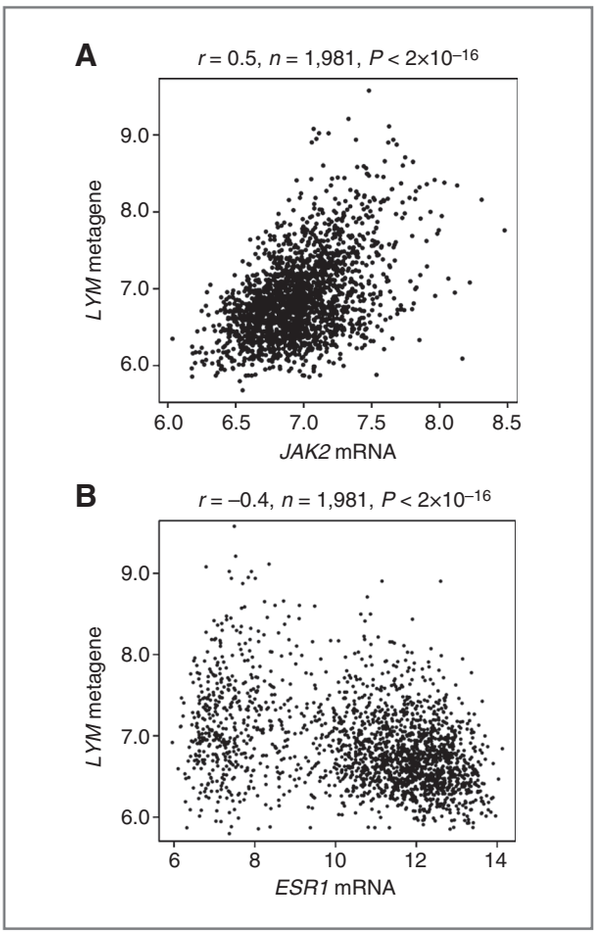

Figure 3. *JAK2* mRNA correlates with the *LYM* metagene signature. A, the average expression of the top-ranked genes of the *LYM* metagene signature (*SASH3*, *CD53*, and *NCKAP1L*) in each tumor sample from METABRIC is shown relative to *JAK2* mRNA. B, the scatter plot for *ESR1*, which is restricted to epithelial cells, is shown for comparison.

Discussion

To our knowledge, this is the first time that a consistent association between increasing *JAK2* mRNA levels and improved breast cancer outcomes has been demonstrated. This association was strongest in the case–control study that matched for variables associated with recurrence. Although the influence of *JAK2* mRNA on survival outcomes in the unmatched public cohorts was predictably not as strong, the remarkably consistent association between higher *JAK2* mRNA and favorable survival is unexpected because JAK2 proteins collaborate with a variety of cytokine receptors that were shown to promote breast cancer growth (3, 4). The association of *JAK2* mRNA with favorable prognosis may reflect a lack of concordance between the levels of *JAK2* mRNA and total JAK2 protein and the active phospho-JAK2 in breast tumor epithelial

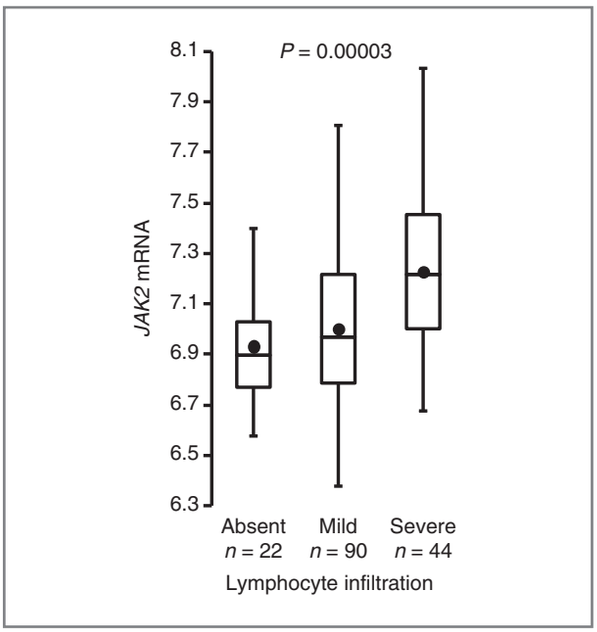

Figure 4. *JAK2* mRNA correlates with levels of tumor-infiltrating lymphocytes. *JAK2* mRNA levels are shown relative to levels of tumor-infiltrating lymphocytes in tumor samples from the favorable prognosis METABRIC integrative cluster 4 that is enriched for an adaptive immune response signature. Box plots, distribution of *JAK2* mRNA values in quartiles. Circle in the box, mean value; horizontal line, median value. The *P* value was calculated using ANOVA.

cells. The association between JAK2 mRNA and favorable prognosis also likely reflects the presence of additional *JAK2*-expressing cell types in the tumor specimens. Consistent with this finding, our previous laser capture microdissection studies demonstrated that *JAK2* mRNA was expressed in both breast tumor epithelial and nonepithelial fractions, but was 8.3 (1.5–44.6)-fold higher in nonepithelial fractions (25). Indeed, we observed a strong correlation between *JAK2* mRNA and levels of tumor-infiltrating lymphocytes and the favorable prognosis *LYM* metagene signature. Our finding that a single gene correlates with a larger biomolecular metagene that is associated with prognosis is reminiscent of the frequent association between single genes and the *PCNA* and *CIN* metagene signatures for proliferation and chromosomal instability (12).

In addition to the present demonstration that the JAK1/2 inhibitor ruxolitinib inhibits CD3-dependent Th1 differentiation, support for a role of JAK2 in T cells is provided by studies demonstrating that JAK2 is involved in the signaling of IL-12 and IFN-γ, key regulators of the tumor-inhibitory Th1 response (24, 26, 27). Furthermore, JAK inhibitors have been shown to impair production of these Th1 cytokines (28) and to inhibit IFN-γ–dependent T-cell trafficking in murine preclinical studies (29). Determining how specific inhibition of the individual JAK family members influences the repertoire and antitumor activities of tumor-infiltrating T cells represents an important area for future investigation. Such studies will provide insights into whether the benefits of inhibiting JAK2 in breast tumor cells outweigh the potential risks of inhibiting tumor-infiltrating T cells.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.P. Miller, J.D. Thorpe, J.D. Beatty, N.D. Urban, C.A. Blau
Development of methodology: C.P. Miller, J.D. Beatty, N.D. Urban
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.P. Miller, A.N. Kortum, J.D. Beatty, C.A. Blau
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.P. Miller, J.D. Thorpe, A.N. Kortum, C.M. Coy, W.-Y. Cheng, T.-H.O. Yang, D. Anastassiou, J.D. Beatty, N.D. Urban, C.A. Blau
Writing, review, and/or revision of the manuscript: C.P. Miller, J.D. Thorpe, A.N. Kortum, C.M. Coy, D. Anastassiou, J.D. Beatty, N.D. Urban, C.A. Blau
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.D. Thorpe, C.M. Coy, J.D. Beatty
Study supervision: C.P. Miller, J.D. Beatty, N.D. Urban, C.A. Blau

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