Short Communication

Cancer Epidemiology, Biomarkers & Prevention

Increased Proliferative Background in Healthy Women with *BRCA1/2* Haploinsufficiency Is Associated with High Risk for Breast Cancer

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Abstract

Previous studies indicated that *BRCA* haploinsufficiency was associated with activation of the EGF receptor (EGFR) signaling pathway and increased proliferative activity in mammary epithelial cells of healthy women. We hypothesized that these processes might be reflected in the expression of serologic soluble EGFR (sEGFR) and thymidine kinase 1 (TK1) activity, which signal the initial and final steps of the proliferative pathway, respectively. We found that healthy carriers of *BRCA1/2* mutations ($n = 80$) showed a significantly higher TK1 activity than age-matched controls ($P = 0.0003$), and TK1 activity was similar in women with *BRCA1* and *BRCA2* mutations ($P = 0.74$). The sEGFR concentration was significantly higher in women with *BRCA1* than in controls and *BRCA2* mutation ($P = 0.013$ and 0.002, respectively). During follow-up, four of 80 *BRCA1/2* mutation carriers developed breast cancer. These women showed a significantly higher TK1 activity and somewhat higher sEGFR concentrations than the other 76 *BRCA1/2* carriers ($P = 0.04$ and 0.09, respectively). All tumors were negative for ovarian hormone receptors, but showed a high EGFR expression. This study was limited by the short-term follow-up (mean, 27 months; range, 5–45), which resulted in a small sample size. Women with *BRCA1* and *BRCA2* mutations that had undergone risk-reducing bilateral salpingo-oophorectomy (BSO) showed significantly lower sEGFR compared with those without surgery ($P = 0.007$ and 0.038, respectively). Larger, prospective studies are warranted to investigate whether TK1 and sEGFR measurements may be useful for identifying healthy *BRCA1/2* carriers with high risk of developing breast cancer; moreover, sEGFR measurements may serve as effective tools for assessing risk before and after BSO. *Cancer Epidemiol Biomarkers Prev; 22(11); 2110–5. ©2013 AACR.*

Introduction

Women with *BRCA1* and *BRCA2* mutations are predisposed to a high risk of breast cancer (57% and 49%, respectively) up to age of 70 years (1). However, there is great variability in the age at diagnosis, and some carriers will not develop the disease during their lifetime. At present, it is unclear how penetrance is related to different biologic factors that affect disease expression. There are no cost effective, valid, and feasible laboratory tests that might be recommended for evaluating the individual risk of breast cancer in a population of *BRCA1/2* mutation carriers.

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doi: 10.1158/1055-9965.EPI-13-0193

BRCA1 and *BRCA2* are tumor-suppressor genes involved in the regulation of cell proliferation. Mutations in these genes were shown to influence growth-related processes. Burga and colleagues (2) studied cultivated primary mammary epithelial cells (PMEC) from healthy *BRCA1* mutation carriers; they revealed a subpopulation of progenitor cells with increased capacity for clonal growth and proliferation compared with normal controls. Later, Martins and colleagues (3) reported that the number of breast epithelial cells positive for the proliferation marker, Ki67, was elevated in normal breast tissue of *BRCA1* mutation carriers. Those findings suggested that in healthy women with *BRCA1* haploinsufficiency, epithelial breast cells possessed enhanced proliferative potential.

Recently, we reported (4) that the proliferative background of human tissues could be assessed with high resolution by measuring the serum activity of thymidine kinase (TK) with a novel procedure (5). Thymidine kinase is a metabolic enzyme that catalyzes ATP-dependent phosphorylation of deoxythymidine to thymidine monophosphate, which is subsequently used in DNA synthesis (6). The enzyme exists in two forms: TK1 (cytoplasmic) and TK2 (mitochondrial). Because TK1 is involved in

DNA synthesis restricted to the S- and G_2-phase (7), it is considered a reliable, sensitive marker of cell proliferation. Dividing cells release TK1 during mitotic exit; this is mediated by the ubiquitin system (8). Thus, TK1 activity can be detected in serum. Recently, novel technology has facilitated the detection of serologic TK1 activity. Women with proliferative breast lesions exhibited higher TK1 activity than women with nonproliferative breast lesions and healthy controls (4).

Previous studies have suggested that deleterious *BRCA1* mutations could cause malignant transformation of breast epithelium; this may be regulated by various growth factors, including EGF (9). EGF receptor (EGFR) expression was high in cultivated PMEC from healthy women with *BRCA1* haploinsufficiency (2). Even a partial loss or inhibition of *BRCA1* was shown to lead to the upregulation of EGFR expression (9), which suggests that mutations in the *BRCA1* gene may be directly or indirectly coupled with high EGFR expression and predispose PMEC to develop EGFR-positive, basal-type breast cancers. It should be emphasized that most studies until now have explored PMEC only from healthy women with BRCA1 mutation. The information on the expression of proliferation markers in healthy carriers of BRCA2 mutation is not available.

The EGFR oncogene encodes a transmembrane tyrosine kinase receptor. The extracellular binding region of this receptor can appear in a soluble form (sEGFR), either by proteolytic cleavage, which releases it from the cell surface (10, 11), or by the alternate transcription of the primary RNA (12). The sEGFR can be detected in serum. The biologic role of sEGFR is largely unknown. Some studies have suggested that it may play a role in regulating EGFR signaling in normal (13) and tumor tissues (11).

Assuming that growth factors are the main drivers of proliferation, in this study, we investigated whether two biomarkers, sEGFR and TK1, which represent the initial and final elements of the proliferative pathway, could be useful for evaluating the proliferative background in healthy carriers of *BRCA1/2* mutations. The results were expected to provide new perspectives in risk assessments of breast cancer. Because bilateral salpingo-oophorectomy (BSO) is known to reduce the risk of breast cancer substantially in *BRCA1/2* mutation carriers (14), we also investigated whether this surgery affected the serum levels of these two biomarkers.

Patients and Methods

A total of 195 women participated in this study, which was approved by the Institutional Ethical Review Board (Registration ID: NCT00855998). All women were recruited from individuals that received genetic counseling from the Genetic Oncology Service. Participants were divided into three groups. The first group included 80 healthy carriers of *BRCA1/2* mutations; 43 had *BRCA1* mutations ($n = 34$ with 185delAG, $n = 8$ with 5382insC, and $n = 1$ with ex18-20dup); and 37 had *BRCA2* mutations ($n = 33$ with 6174delT, $n = 2$ with 1vs+1G>A, $n = 1$ with 8675del, and $n = 1$ with G.6024dupG). The second group included 80 healthy female blood donors that had not been tested for *BRCA1/2* mutations. Women from the second group were matched by age ($\pm$ 2 years) with women in the first group. The prevalence of *BRCA1/2* mutations is about 2.5% in women from an Ashkenazi Jewish population (15); therefore, two individuals were estimated to have *BRCA1/2* mutations among the control group. This fraction (2 of 80) was not considered sufficient to influence the results. Exclusion criteria for the two groups were pregnancy and breast feeding. A third group included 35 healthy carriers of *BRCA1/2* mutations; 14 had *BRCA1* mutations ($n = 13$ with 185delAG and $n = 1$ with 5382insC) and 21 had *BRCA2* mutations ($n = 18$ with 174delT, $n = 2$ with 1vs+1G>A, and $n = 1$ with 6174delT). This group had undergone preventive BSOs before the enrollment (mean time since surgery, 4 years; range, 1–11 years).

All participants were fully informed about the study, provided written consent, and donated a blood sample. Serum was isolated, aliquoted, stored for 20 to 30 days at -80°C, and analyzed after blind coding.

Serum TK1 activity was measured with a highly sensitive, colorimetric ELISA kit (DiviTum; Biovica International AB) as outlined previously (4). The analytic sensitivity, defined as the minimum detectable dose distinguishable from zero by 2 SDs, was $\leq$5 Du/L. The coefficients of variation within- and between-assays, measured at 100 Du/L, were 7.0% and 11.3%, respectively. Serum sEGFR concentrations were determined with the commercially available human EGFR immunoassay (Quantikine®, R&D systems). Results are expressed as ng/mL. The analytic sensitivity was 0.014 ng/mL. The coefficients of variation within- and between-assays, measured at 5 ng/mL, were 3.7% and 10.0%, respectively. Serum follicle-stimulating hormone (FSH) concentrations were assayed according to the manufacturer's specifications using the Architect System (Abbott). The FSH concentrations were used to assist in determining the women's menopausal status. The criteria used to assign postmenopausal status were: age >60 years, BSO, or FSH concentration >26 IU/L (reference interval for Architect System).

Statistical analysis

Measurements of markers are expressed as the mean, median, and interquartile range (IQR). The distribution of data in the DiviTum and Quantikine assays were found to be asymmetric, with high kurtosis, therefore, the nonparametric Wilcoxon signed-rank and Wilcoxon rank-sum tests were used for comparisons. The Spearman coefficient (r) was used to measure correlations between variables. Statistical calculations were conducted with SPSS, version 10 for Windows. A P value less than 0.05 was considered significant.

Results and Discussion

The group of healthy *BRCA1/2* mutation carriers and the matched control group had a mean age of 37 years (range, 20–65). The group that had undergone preventive BSO had a mean age of 50 years (range, 32–66), significantly older than the other two groups ($P < 0.0001$ for both). TK1 activity was not correlated with age and FSH ($n = 195$; $r = 0.09$, and 0.04, respectively). In accordance with previous observations (16), we found weak negative correlation of serum EGFR concentrations with age ($r = -0.22$) and concentrations of FSH ($r = -0.23$). The analysis of TK1 activity with regards to menopausal status showed that TK1 was not differed in premenopausal and postmenopausal women (medians, 31 vs. 38; $P = 0.25$). However, sEGFR concentrations were significantly higher in premenopausal than in postmenopausal women (medians, 48.4 vs. 46.1; $P = 0.004$).

As may be expected, we did not find a significant association of TK1 activity with age, menopausal status, and FSH concentrations. It seems that there are some other conditions that could contribute to the variability of TK1 results. A fraction of the deviating higher TK1 levels may be due to compensatory cell division after cell destroying disease, for example, certain viral infections (17) or physical trauma, for example, surgery (18). The normalization of TK1 levels after these conditions may last for 6 to 8 weeks. In future studies, transient increases can be eliminated by establishing the stable baseline of TK1 for each individual by repeated serum sampling.

In this study, we found that *BRCA1/2* haploinsufficiency in healthy women was associated with significant changes in TK1 activity and sEGFR concentration. The activity of TK1 in the serum of *BRCA1/2* mutation carriers was significantly higher (Table 1 and Fig. 1A; medians, 41 vs. 20; $P = 0.0003$) than in that of age-matched healthy controls. In a separate analysis, both *BRCA1* and *BRCA2* mutation carriers showed a significantly higher TK1 activity compared with matched controls (medians, 44 vs. 18; $P = 0.006$ and medians, 38 vs. 21; $P = 0.011$).

The sEGFR concentration was not significantly different between carriers and matched controls (Table 1, Fig. 1B; medians, 48.5 vs. 48.0; $P = 0.22$). However, when the sEGFR distributions were analyzed by genotype, the sEGFR concentration was significantly higher in women with *BRCA1* mutations compared with controls (median, 50.2 vs. 48.1, $P = 0.013$), with no difference between the *BRCA2* and control groups (medians, 46.9 vs. 48.0; $P = 0.27$).

To avoid the confounding effect of age, we compared TK1 activity and sEGFR concentrations in age-matched women with *BRCA1* and *BRCA2* mutations. Analysis of 31 pairs showed significantly higher concentrations of sEGFR in women with BRCA1 mutation compared with those with BRCA2 mutation (medians, 49.8 vs. 46.8; $P = 0.002$), with no difference in the TK1 activity (medians, 42 vs. 32; $P = 0.74$). The age-matched carriers of *BRCA1* and *BRCA2* mutations did not differ in concentrations of FSH (medians, 5.4 vs. 5.0; $P = 0.58$).

Thus, healthy women with *BRCA1* haploinsufficiency were characterized by elevated levels of two serum markers related to proliferation. These data were consistent with previous observations of high EGFR and Ki67 expression in PMEC obtained from healthy carriers of *BRCA1* mutations (2, 3). Those cells showed a high capacity for clonal growth and proliferation. Those authors related these properties to upregulation of the EGFR pathway. We also showed that *BRCA2* carriers had an elevated serum TK1 activity, but unlike *BRCA1* carriers, they had low sEGFR levels. Thus, serologic sEGFR, like tissue EGFR expression, was significantly associated with *BRCA1* haploinsufficiency in healthy women.

We followed 80 *BRCA1/2* carriers for a mean of 27 months (range, 5–45 months). Of these, four developed breast cancer with mean time to diagnosis of 19 months (range, 13–22 months); 3 had a founder *BRCA1* mutation (185delAG), and 1 had a founder *BRCA2* mutation (6174delT). The TK1 activity in this small set was significantly higher compared with the other 76 women of this group (Table 1, Fig. 1A; medians, 77 vs. 39; $P = 0.04$). The

Table 1. Levels of serum TK1 and sEGFR in healthy women identified as noncarriers (control) or carriers ($^+$) of *BRCA1/2* mutations

		Serum TK1 activity (Du/L)			sEGFR concentration (ng/mL)		
Study group	***N***	**Mean**	**Median**	**IQR**	**Mean**	**Median**	**IQR**
Matched healthy (control)	80	46	20	12–35	47.6	48.0	42.3–53.5
Matched healthy BRCA1/2$^+$	80	70	41	23–71	49.8	48.5	45.4–52.7
BRCA1$^+$	43	67	44	21–67	51.9	50.2	47.7–54.6
BRCA2$^+$	37	74	38	23–81	47.4	46.9	44.1–50.1
BRCA1/2$^+$ that developed breast cancer[a]	4	90	77	65–130	52.3	51.7	48.7–56.6
Healthy BRCA1/2$^+$ BSO$^+$	35	58	41	23–71	44.6	45.9	41.1–48.6
BRCA1$^+$	14	55	50	30–66	45.9	46.7	40.7–50.3
BRCA2$^+$	21	61	37	15–68	43.8	44.9	41.5–47.6

[a]Individuals from the healthy *BRCA1/2*$^+$ group.

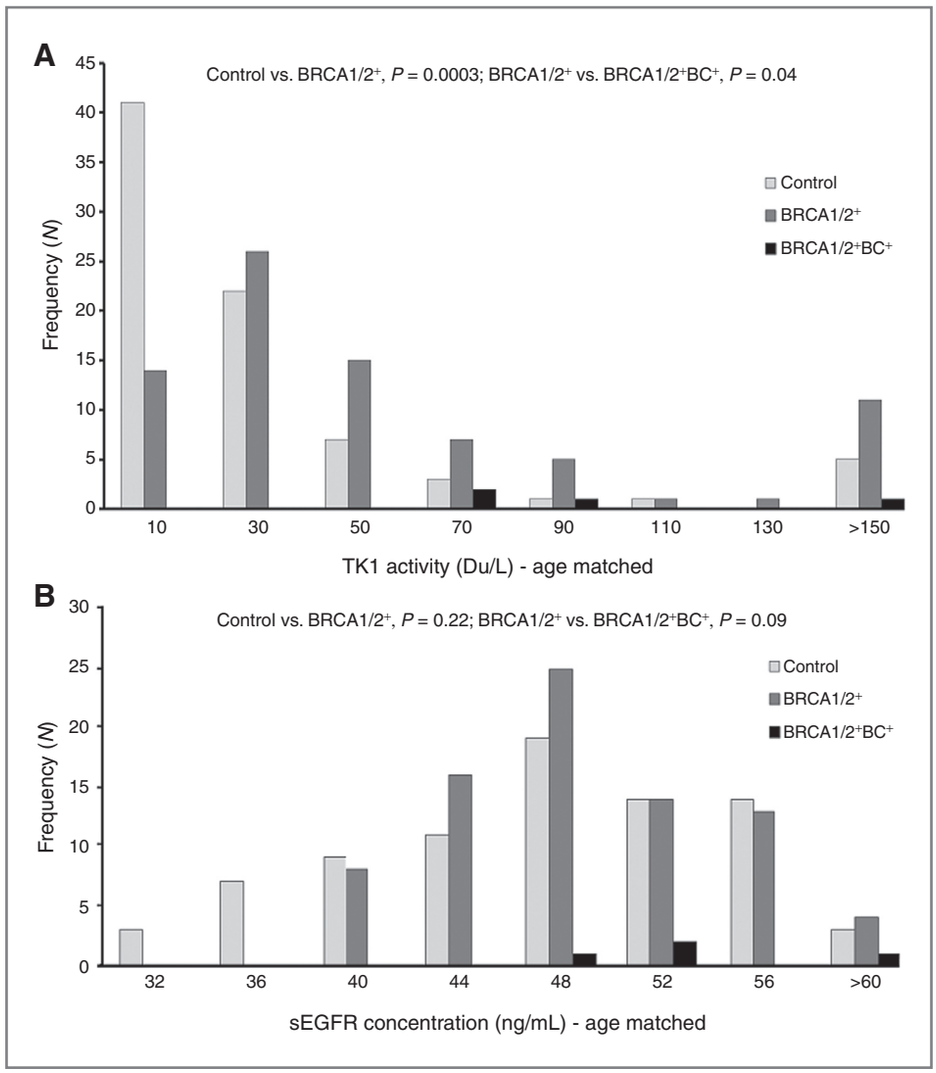

Figure 1. Serum TK1 activity and sEGFR concentrations in healthy women identified as noncarriers or carriers of *BRCA1/2* mutations. Comparison of (A) TK1 activity or (B) sEGFR concentrations in age-matched groups of healthy control women, healthy carriers of *BRCA1/2* mutations (BRCA1/2$^+$), and carriers from the healthy BRCA1/2$^+$ group that subsequently developed breast cancer (BRCA1/2$^+$BC$^+$). *P* values were derived from Wilcoxon signed-rank and Wilcoxon rank-sum tests.

concentration of sEGFR ng/mL was also higher in women with breast cancer compared with those without it, but the difference did not reach any statistical significance (Table 1, Fig. 1B; medians, 51.7 vs. 48.5; $P = 0.09$). Interestingly, all four cases were found to be triple-negative breast cancer (TNBC) and positive for EGFR. This confirmed results from previous studies, which showed an inverse relationship between hormone receptors and EGFR expression (19). These findings suggested a possible association between increased serologic sEGFR in women with *BRCA1/2* that are at risk of breast cancer and EGFR overexpression later observed in these women with breast cancer.

Thus, the levels of two serum biomarkers, TK1 and sEGFR, in the population of healthy *BRCA1/2* mutation carriers allowed the identification of individuals with increased proliferative background, who were at high risk for developing breast cancer. It should be emphasized that the short-term follow-up of this study resulted in a small sample size of breast cancer cases; this was an important limitation of this study, and it merits further investigation.

We analyzed the relationship between sEGFR and TK1 in the different subgroups, and found that they were highly correlated in carriers of *BRCA2* mutations ($n = 37$; $r = 0.60$), but not in carriers of *BRCA1* mutations ($n = 43$; $r = 0.09$). These findings may indicate that the regulation of proliferative signals may be different in women with *BRCA1* and *BRCA2* haploinsufficiency. We speculated that the high-serum sEGFR expression in the *BRCA1* mutation group was consistent with a mechanism of constitutive EGFR activation, but in the *BRCA2* group, the EGFR pathway seemed to be more responsive to external signals that led to DNA synthesis.

We also considered the possibility that TK1 and sEGFR might be associated with the observation that BSO reduced the risk of breast cancer. Analysis of the TK1 distribution in *BRCA1/2* mutation carriers that underwent

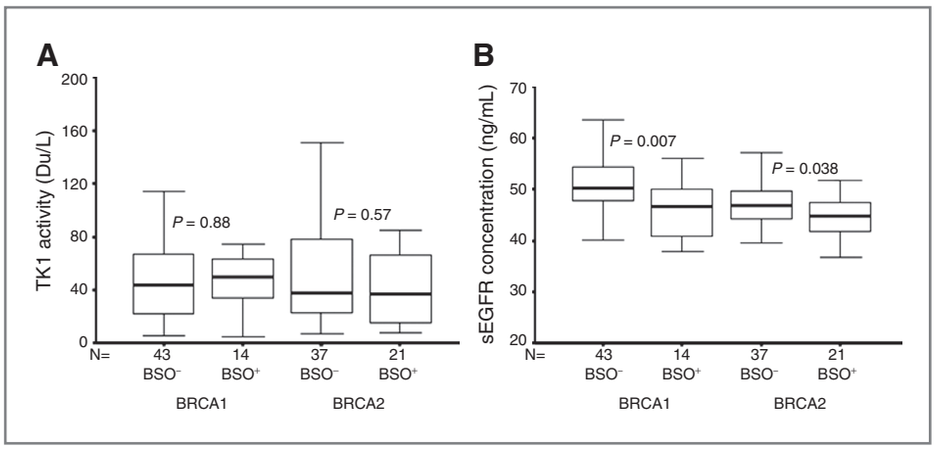

Figure 2. Comparison of (A) TK1 activity or (B) sEGFR concentration in healthy carriers of either *BRCA1* or *BRCA2* mutations that did (⁺) or did not (⁻) undergo preventive BSO. Each box plot shows the median (heavy line), quartiles (box ends), and extreme values (whiskers) within the category shown. *P* values were derived from Wilcoxon rank-sum test.

BSO did not show a normalization of TK1 activity (Table 1, Fig. 2A). Instead, TK1 activity remained increased in both *BRCA1* and *BRCA2* carriers compared with controls (Table 1; medians, 50 vs. 20; $P = 0.007$ and medians, 37 vs. 20; $P = 0.03$).

The sEGFR (Table 1, Fig. 2B) concentration in women with *BRCA1/2* mutations that had undergone BSO was significantly lower compared with those without preventive surgery (medians, 45.9 vs. 48.5; $P = 0.0002$) and controls (medians, 45.9 vs. 48.0; $P = 0.022$). Furthermore, in both the *BRCA1* and *BRCA2* subgroups, those that underwent BSO showed reduced sEGFR levels compared with those without BSO (Fig. 2B; medians, 46.7 vs. 50.2; $P = 0.007$ and medians, 44.9 vs. 46.9; $P = 0.038$, respectively). Among women carriers of BRCA1/2 mutation, those who had surgically induced menopause have significantly lower concentrations of sEGFR compared with those in natural menopause (medians, 46.1 vs. 49.5; $P = 0.016$), with about similar concentrations of FSH (medians, 60.7 vs. 62.3; $P = 0.63$). We speculate that the effect of menopause induced by surgery could be stronger compared with natural menopause, which develops more slowly.

A well-known etiology for breast cancer development involves ovarian hormones mediating biologic effects through hormone receptors. However, most carriers of *BRCA1* and, to a lesser extent *BRCA2*, develop TNBC. Laboratory investigations with cultivated PMEC from healthy women with *BRCA1* haploinsufficiency showed that these cells lacked estrogen receptors (2). Nevertheless, BSO was effective in reducing the risk of breast cancer in both *BRCA1* and *BRCA2* mutation carriers (14); this suggested that for these women, the risk reduction was most likely associated with decreased ovarian-hormone exposure. The decreased levels of sEGFR after BSO in women with *BRCA1* and *BRCA2* mutations observed in our study might indicate that sEGFR levels were hormonally regulated in both mutation groups.

In our study, we did not find any significant decrease of TK1 levels in women who had undergone BSO, suggesting its nonovarian origin. Because increased EGFR expression was shown both in breast cancer (20) and normal mammary cells of BRCA1 mutation carriers (2), it is most probably that breast tissue is the main origin for sEGFR. However, the generation of TK1 and sEGFR by other than breast organs cannot be excluded. Decreased ovarian hormone exposure after BSO may lead to the reduction of sEGFR production in breast tissue.

In conclusion, our results indicated that measurements of TK1 and sEGFR may be useful for identifying women with *BRCA1/2* haploinsufficiency that are at high risk of developing breast cancer, and for whom risk-reduction interventions could be recommended. Future prospective studies are warranted to explore the associations of TK1 activity and EGFR levels with other important factors associated with risk of breast cancer, such as body mass index, history of parity, breast feeding, use of hormones such as pills, hormone-replacement therapy as well as breast density to develop a model for breast cancer risk prediction in BRCA1/2 mutation carriers.

Disclosure of Potential Conflicts of Interest

S. Gronowitz is employed as Research Manager for Biovica International AB and has ownership interest (including patents) in the same. T. Peretz is a consultant/advisory board member of Biovica International AB. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: L. Kadouri, T. Peretz
Development of methodology: S. Gronowitz, T. Peretz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Nisman, L. Kadouri, T. Allweis, B. Maly, T. Peretz
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Kadouri, B. Maly, T. Hamburger, S. Gronowitz
Writing, review, and/or revision of the manuscript: B. Nisman, L. Kadouri, T. Allweis, B. Maly, T. Peretz
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Inventor of high-sensitivity TK assay used and supervising assays, and compiling data from analyses: S. Gronowitz

Received February 22, 2013; revised August 5, 2013; accepted August 8, 2013; published OnlineFirst August 21, 2013.

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