Stromal-derived interleukin-6 drives epithelial-to-mesenchymal transition and therapy resistance in esophageal adenocarcinoma

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Esophageal adenocarcinoma (EAC) has a dismal prognosis, and survival benefits of recent multimodality treatments remain small. Cancer-associated fibroblasts (CAFs) are known to contribute to poor outcomes by conferring therapy resistance to various cancer types, but this has not been explored in EAC. Importantly, a targeted strategy to circumvent CAF-induced resistance has yet to be identified. Using EAC patient-derived CAFs, organoid cultures, and xenograft models, we identified IL-6 as the stromal driver of therapy resistance in EAC. IL-6 activated epithelial-to-mesenchymal transition in cancer cells, which was accompanied by enhanced treatment resistance, migratory capacity, and clonogenicity. Inhibition of IL-6 restored drug sensitivity in patient-derived organoid cultures and cell lines. Analysis of patient gene expression profiles identified ADAM12 as a noninflammation-related serum-borne marker for IL-6-producing CAFs, and serum levels of this marker predicted unfavorable responses to neoadjuvant chemoradiation in EAC patients. These results demonstrate a stromal contribution to therapy resistance in EAC. This signaling can be targeted to resestitivize EAC to therapy, and its activity can be measured using serum-borne markers.

IL-6 | tumor stroma | therapy resistance | esophageal adenocarcinoma | epithelial-to-mesenchymal transition

Esophageal cancer has a poor prognosis and currently ranks sixth in cancer-related mortality (1, 2). A steep increase in the incidence of the esophageal adenocarcinoma (EAC) subtype has been observed in Western countries (2). Patients eligible for curative treatment typically receive neoadjuvant chemoradiation therapy, followed by surgery (3). The efficacy of this regimen is modest, indicating a need to identify the mechanisms that contribute to therapy resistance.

Research on therapy resistance has centered on tumor cell-intrinsic properties, but it is increasingly clear that the tumor microenvironment (TME) is important for this as well (4). Cancer-associated fibroblasts (CAFs) comprise the majority of the TME and are suspected to exert tumor-promoting activities by their mechanical contributions to the stroma, as well as by the secretion of cytokines (5). The presence of CAFs, as determined by expression of smooth muscle actin (α-SMA), is associated with poor survival in many solid malignancies (6–9), but the exact tumor-promoting activities of these cells vary between cancer types (10). The specific contributions of CAFs depend on the cytokines produced.

IL-6 is primarily known for its role in inflammation, which can result from exposure to anticancer drugs and ionizing radiation. IL-6 may also be expressed in the absence of therapeutic stress (11, 12). In various cancer types, both the tumor cells and CAFs can produce IL-6 (13–16). The tumor-promoting activities of IL-6 are manifold and include the evasion of growth suppression by regulatory T cells (17), mediating resistance against cell death (18, 19), increasing aggressiveness of tumor cells (20, 21), and mediating tumor invasion and metastasis (22–24). Also, stroma-derived IL-6 is dysregulated in the metaplasia–dysplasia–EAC sequence (25).

Surprisingly little is known about the role of CAFs in EAC (8, 9, 26, 27). In this study, tumor cells and EAC-associated fibroblasts were isolated and used to identify a mechanism of resistance against currently applicable treatment regimens in EAC. Stromal IL-6 was identified as the molecule driving this resistance, and targeting IL-6 resulted in resensitization of tumor cells to chemoradiotherapy.

Significance

We found that cancer-associated fibroblasts, the most abundant noncancer cell type in esophageal cancer tissue, contribute to the resistance of tumors against currently applicable treatments. These cancer-associated fibroblasts do so by producing and secreting IL-6. IL-6 induces a mesenchymal, resistant tumor cell state. Inhibition of IL-6 reverted the mesenchymal cell state and resensitized tumor cells to therapy. Serum-borne proxies for the presence of IL-6–producing fibroblasts were identified and found to be predictive for the response to neoadjuvant therapy.

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Results

Patient-Derived EAC-Associated Fibroblasts Confer Resistance to Chemotherapy and Radiotherapy. To investigate a possible contribution of CAFs to resistance against conventional chemotherapy and radiation therapy, primary EAC-associated fibroblasts were isolated from resected specimens from patients who received paclitaxel with carboplatin and radiation [the Chemoradiotherapy for Oesophageal cancer followed by Surgery Study (CROSS) regimen] (3) (SI Appendix, Fig. S1A). Cells were stained with anti–α-SMA to confirm their activated myofibroblast-like state (SI Appendix, Fig. S1B). Neoadjuvant chemoradiation is the standard of care in many western European countries and the United States. Therefore, CAFs derived from resection specimens will often have been exposed to this treatment. Two previously established EAC cell lines, OE19 and OE33, were treated with carboplatin, paclitaxel, or radiation in the absence or presence of CAF supernatant. CAF supernatant was found to confer resistance against the applied therapeutics (Fig. 1 A and B), as well as other clinically relevant agents such as 5-fluorouracil (5-FU), cisplatin, and eribulin (SI Appendix, Fig. S1 C and D). Of note, cells that survived the therapy showed a shift in morphology (Fig. 1C). Tumor cell sensitivity to chemotherapeutics was not influenced by the addition of Iscove’s Modified Dulbecco’s Medium (IMDM) (CAF medium) (SI Appendix, Fig. S1 E and F).

Stromal CAF-Secreted IL-6 Drives Therapy Resistance. To assess the association of the candidate cytokine with patient outcome, we performed survival analysis on the publicly available TCGA gene expression set containing pretreated resected esophageal cancer specimens (28). Samples from EAC (TCGA-EAC) patients were dichotomized by median IL6, CCL2, or HGF expression. A significant association with survival was found for only IL6 (SI Appendix, Fig. S6d). To functionally address which cytokine was responsible for the CAF-induced treatment resistance, recombinant IL-6, CCL2, HGF, or CAF supernatant preincubated with the pertinent neutralizing antibodies was used in cell viability assays on two primary EAC cultures receiving carboplatin, paclitaxel, or radiation (Fig. 2 A–F). Of the candidates tested, IL-6 most consistently affected therapy resistance. Next, we examined whether IL-6 was specifically produced by CAFs rather than by tumor cells. Indeed, ELISA on cell supernatant. Graphs show means ± SEM, normalized to t = 0, n = 3. P values were determined by two-way ANOVA and Bonferroni correction. (B) Same as for A, using the OE33 cell line. (C) OE19 and OE33 cell lines were cultured in unconditioned or 081RF supernatant-supplemented medium (081RF sup), treated with 256 pM paclitaxel or control for 168 h, and morphology was assessed by phase-contrast microscopy. (Scale bar: 100 μm.) (D) Cell viability was determined on 007B and 031M cells which were incubated with the indicated chemotherapeutics and parenthesized concentrations in unconditioned control (ctr) medium (gray lines) or medium supplemented with 081RF supernatant (1 in 4 diluted) (colored lines). Graphs show means ± SEM of data normalized to t = 0, n = 3. P values were determined by two-way ANOVA and Bonferroni correction.

To investigate whether IL-6 secreted by CAFs can activate its promoting properties of the EAC stroma are largely driven by CAF-secreted, biologically active IL-6. EOB19 and OE33, were treated with carboplatin, paclitaxel, or radiation (Fig. 1B). Neoadjuvant chemoradiation is the standard of care in many western European countries and the United States. Therefore, CAFs derived from resection specimens will often have been exposed to this treatment. Two previously established EAC cell lines, OE19 and OE33, were treated with carboplatin, paclitaxel, or radiation in the absence or presence of CAF supernatant. CAF supernatant was found to confer resistance against the applied therapeutics (Fig. 1 A and B), as well as other clinically relevant agents such as 5-fluorouracil (5-FU), cisplatin, and eribulin (SI Appendix, Fig. S1 C and D). Of note, cells that survived the therapy showed a shift in morphology (Fig. 1C). Tumor cell sensitivity to chemotherapeutics was not influenced by the addition of Iscove’s Modified Dulbecco’s Medium (IMDM) (CAF medium) (SI Appendix, Fig. S1 E and F).

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To investigate whether IL-6 secreted by CAFs can activate its canonical pathway in the cancer cells, 007B and 031M cells were stimulated with CAF supernatant, which resulted in STAT3 phosphorylation. The specificity of this effect was confirmed using IL-6-neutralizing antibody (Fig. 2F). See SI Appendix, Fig. S7 for full membranes. These data suggest that the tumor-promoting properties of the EAC stroma are largely driven by CAF-secreted, biologically active IL-6.

CAF-Derived IL-6 Induces Epithelial-to-Mesenchymal Transition. From the cell viability experiments, a marked change in morphology in the surviving cells was apparent (see Fig. 1C). To identify the
changes were confirmed at the protein level by flow cytometry, which showed increased expression of EMT markers C-X-C chemokine receptor type 4 (CXCR4) and VIM and decreased expression of epithelial-related genes human epidermal growth factor receptor 2 (ERBB2), cluster of differentiation 24 (CD24), integrin beta-1 (CD29), and epithelial cell adhesion molecule (EPCAM) (SI Appendix, Fig. S9 E–I). We and others have previously found human epidermal growth factor receptor 3 (HER3/ERBB3) to be a marker for epithelial cell identity, and this protein was also down-regulated following exposure to IL-6 (30–32). Analysis of the kinetics of EMT onset in response to CAF supernatant revealed this EMT to take place within several days, a time frame in line with the induction of chemoresistance (SI Appendix, Fig. S10). We take these data to show that IL-6 activates EMT in cancer cells and that this is the mechanism through which resistance against commonly used chemotherapeutics is conferred by the stroma.

IL-6-Induced EMT Is Accompanied by an Enhanced Migratory and Clonogenic Capacity. To study the functional effects of the up-regulated EMT markers in addition to the morphological changes, transwell migration assays were performed, and they showed an enhanced migratory capacity following exposure to IL-6 events responsible for this, we performed gene set enrichment analysis (GSEA) on the TCGA-EAC dataset using gene sets for biological programs associated with such phenotypic transitions. Samples were dichotomized by median IL6 expression, and a significant association was found for a merged set of two previously published epithelial-to-mesenchymal transition (EMT) signatures and for a stromal infiltration gene set. Additionally, low-IL6-expressing tumors associated with an epithelial signature (SI Appendix, Fig. S6 D–F).

To further ascertain EMT as the mechanism responsible for IL-6–induced therapy resistance, primary cells were cultured with CAF supernatant, IL-6, or CAF supernatant preincubated with IL-6–neutralizing antibody, and morphology was monitored by microscopy. The induction of a mesenchymal morphology was apparent (Fig. 3A). These morphological changes were also observed using supernatant from treatment-naive CAFs (SI Appendix, Fig. S8). Using early-passage EAC organoids in the same experimental setup, cells in the IL-6–containing conditions were observed to migrate out of the organoid structures and the Matrigel (Fig. 3B). To characterize and quantify these observations at the molecular level, EMT markers were measured by transcript analysis, and increased expression of zinc finger E-box-binding homeobox 1 (ZEB1), vimentin (VIM), snail family transcriptional repressor 2 (SNAI2), and N-cadherin (CDH2) was found in the cultures exposed to IL-6. Epithelial markers E-cadherin (CDH1), and cytokeratin 19 (KRT19) were decreased (SI Appendix, Fig. S9 A–F).
(Fig. 3 C and D). Furthermore, concomitant with the up-regulation of EMT markers, cancer stem cell (CSC) markers CD44/CD44, prominin-1 (PROM1/CD133), and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5/LGR5) were increased (SI Appendix, Fig. S9 G–L). This was accompanied by an increased clonogenicity in limiting dilution assays (Fig. 3D) and implies that stroma-derived IL-6 drives many of the EMT-associated biological programs that are known to contribute to poor outcome in cancer.

To allow an assessment of the contributions of IL-6 signaling to tumor growth in vivo, the problem of species incompatibility between IL-6 and its receptor needed to be addressed (33). To allow mouse-human transsignaling and human-human autocrine signaling, we generated 031M cells expressing the mouse IL-6 receptor (mIL-6Ra) and human IL-6 ligand (hIL-6; and empty vector), respectively. These cells were injected in immunodeficient mice, and tumor growth was observed only from cells expressing mIL6Ra or hIL-6 (SI Appendix, Fig. S11), confirming that IL-6 signaling also contributes to clonogenicity in vivo.

**Stroma-Derived IL-6 Confers Resistance to Radiochemotherapy in EAC Patients.** In current clinical practice, patients diagnosed with EAC eligible for curative therapy receive neoadjuvant carboplatin, paclitaxel, and fractionated radiation [the CROSS regimen (3)]. To study whether CAF-derived IL-6 can confer resistance to such a triple-modality regimen, we modeled this treatment by determining the combined doses of the regimen components that allow for a near-complete cell killing, similar to the encouraging but often incomplete responses seen in patients. Primary EAC cells were given one dose of carboplatin and paclitaxel and subsequently received seven radiation doses of 1 Gy. Colony formation was assessed, and efficient outgrowth was observed only in the presence of IL-6 (Fig. 4 A and B). To confirm this response in a model system more representative of human disease, early-passage PDX-derived EAC organoids (which underwent clonal selection only during graft expansion, which ensured the tumor cell origin of the organoids) were subjected to the same treatment, and the ability to passage the cultures after triple-modality treatment was determined (Fig. 4C). Organoid outgrowth following passaging was observed only in cultures exposed to IL-6 and did not occur in the control or IL-6–neutralized conditions.

Having identified the molecule responsible for EMT-associated therapy resistance in EAC cells exposed to triple-modality treatments, a logical step would be to measure this cytokine in the serum of patients and correlate it to response, yielding a predictive marker that can predict neoadjuvant treatment outcome. Serum samples from 82 EAC patients before start of neoadjuvant chemoradiotherapy were analyzed for IL-6, and no significant difference was found between patients grouped by tumor response (Mandard score; Fig. 4D), probably reflecting the association of IL-6 with numerous inflammatory conditions. Instead, we identified ADAM12 by correlative gene expression analyses as a more specific marker for stromal CAFs (SI Appendix, Fig. S6G). Its expression was associated with poor prognosis (SI Appendix, Fig. S6F). ADAM12 levels in the serum are known to correlate with disease stage in lung cancer (34–36), and its expression is mostly confined to stromal cells in gastrointestinal cancers (37, 38). Measuring serum ADAM12 in these patients revealed a strong correlation to circulating IL-6 (Fig. 4E). Of note, high circulating ADAM12 levels significantly correlated with poor response to chemoradiation (Mandard score of 3–4) in EAC patients (Fig. 4F). Treatment of CAFs with recombinant IL-6 or blocking IL-6 ligand with antibody did not induce or affect ADAM12 secretion, but addition of recombinant TGF-β did (Fig. 4G). This confirms that ADAM12 is a feature of highly activated CAFs and that this activation likely does not result from autocrine IL-6 signaling. Future work will have to validate these findings in other cohorts and confirm whether ADAM12 is indeed an accurate measure of IL-6–producing CAFs in the activated EAC stroma and a predictive marker for currently applicable treatments against EAC.

![Fig. 4.](https://www.pnas.org/doi/10.1073/pnas.1820459116)
Discussion

Despite the advent of multimodality treatment, the overall survival of EAC patients remains poor. This is mainly due to therapy resistance and aggressive tumor growth leading to the early onset of metastatic disease. Previous studies on therapy resistance mechanisms in EAC mostly focused on tumor cell-intrinsic mechanisms, but it is now well-established that the stroma plays a central role as well (8, 9, 26, 27). Here we investigated how EAC-associated fibroblasts impact therapy resistance and identified IL-6 as a mediator of this resistance.

Upon treatment with recombiant or CAF-derived IL-6, we observed that the tumor cells obtained a mesenchymal phenotype that was accompanied by increased migration. Indeed, EMT is associated with poor disease outcome, attributed to the enhanced migratory capacity of the cells that is thought to contribute to metastasis (30–41). Importantly, EMT was recently described to confer resistance to cytotoxic agents, which underscores our observation of EMT-induced chemoresistance (42, 43). In addition, IL-6 enhanced clonogenicity in vitro and in vivo and up-regulated the expression of CSC markers, which has been linked to EMT as well (44). This could well explain the resistance against a broad range of cytotoxic agents and radiation that has been attributed to stemlike cancer cells. Furthermore, IL-6 has previously been linked to tumor invasion and metastatic progression (45), and here we showed that this is, at least in part, caused by the induction of EMT.

The apparent readiness of EAC cells to undergo EMT in response to extrinsic cues and stresses offers a unique opportunity to study the kinetics of this transition. In doing so, we found that the onset of EMT in response to CAF supernatant already occurred within several days, explaining the resistance observed in the cell viability assays. Also, this rapid transition to a mesenchymal state is in line with the notion that therapy resistance is instructed by IL-6 in the majority of the EAC tumor cell population, rather than selection of a limited number of clones.

The ability to predict treatment response by means of a non-invasive serum marker would be of immense clinical value. IL-6 is primarily involved in inflammatory processes, and its serum levels are elevated in patients suffering from asthma and rheumatoid arthritis (46, 47). Furthermore, inflammation has a great influence on the onset and progression of various cancer types, including EAC (48–50). Consequently, IL-6 is likely not a very specific serum marker for treatment response in EAC, and we found no correlation with IL-6 serum levels and response to neoadjuvant treatment. Instead, we identified ADAM12 as one of the stromal genes most strongly correlating with IL6. ADAM12 is known to exist in the circulation (51), and indeed, ADAM12 serum levels in pretreated EAC patients from our hospital differed significantly between patients dichotomized by response to neoadjuvant treatment. This suggests that the amount of “activated stroma” can be seen as a measure, or predictor, of therapy response. This could also be of use for specific stroma-targeting strategies, including the direct targeting of CAFs (52). However, considerable overlap exists in ADAM12 levels between the two groups, and for use in clinical decision making, additional markers should be included. Also, it should be noted that the stroma is not the only factor to impact therapy response and that other compartments and signals contribute to heterogeneous responses that therefore cannot be explained by serum-borne proxies of stromal activation status.

In summary, our data elucidate the mechanisms through which the tumor stroma contributes to therapy resistance in EAC and demonstrate that ADAM12 serum levels predict treatment outcome in patients. Given these findings and the availability of relevant FDA-approved IL-6-targeting agents, we propose that addition of such agents to currently applicable regimens should be strongly considered.

Materials and Methods

Informed Consent Procedure. Signed informed consent was obtained for all patients included in the BIOES biobank according to procedures approved by the Academic Medical Center’s ethical committee (MEC 01/288/08.17.1042). This consent covers all procedures described in this paper, including the collection of clinical data, tissue, and blood for marker analysis and expansion as xenografts and in vitro cultures.

Establishment of Primary Cancer-Associated Fibroblasts. Primary EAC-associated fibroblasts were established from resected tumor specimens of EAC patients treated at the Academic Medical Center (Amsterdam, The Netherlands) according to the CROSS regimen (carboplatin, paclitaxel, and radiation) (3). Fresh tumor pieces were washed three times for 5 min with PBS containing penicillin (100 units/ml), streptomycin (500 μg/mL), and gentamicin (5 μg/mL); cut into small pieces; and resuspended in DMEM containing Liberase and DNase for 45 min. Subsequently, cells were resuspended, passed through a 100-μm cell strainer, and spun down. Cells were resuspended in IMDM medium containing 8% FBS, 1-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (500 μg/mL) and plated in a T25 culture flask. After 48 h, all nonadherent cells were discarded by washing with PBS. Cells were maintained according to standard culture conditions, and upon reaching 80% confluency, cell sorting by negative selection (EPICAM-negative) was performed to obtain a pure fibroblast culture. Antibodies are listed in SI Appendix, Table S1. The primary EAC-associated fibroblasts exposed to neoadjuvant chemoradiation used are as follows: AMC-EAC-0181RF (0181RF) and AMC-EAC-2433RF (2433RF). Treatment-naive primary fibroblasts are AMC-EAC-P1178F (1178F) and AMC-EAC-2889BF (2889BF).

Establishment of Primary Tumor Cell Cultures. Primary cultures were established as described before from PDXs (53). Briefly, tumor material of patients diagnosed with EAC in the Academic Medical Center (Amsterdam, The Netherlands) was obtained in accordance with approval by the institute’s ethical committee (MEC 01/288/08.17.1042) (54). The tumor material was expanded in NOD.Cg-Prkdcscid/lzg–/–Igσ–/–SjI-295N-Tg16925)NSG–/–immunodeﬁcient mice. Ethical approval was obtained (LEK102774), and the NSG mice were bred and maintained at the central laboratory facility according to current guidelines. Established primary cultures were maintained in IMDM medium containing 10% FBS, 1-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (Sigma-Aldrich), 5 mM Hepes (Life Technologies), 0.15% Δ-glucone (Sigma-Aldrich), 100 μM β-mercaptoethanol (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), 2 μg/mL heparin (Sigma-Aldrich), and 1:1,000 trace elements B and C (Fisher Scientiﬁc). The primary EAC cultures used are as follows: AMC-EAC-0078E (0078), which was established from a pretreatment biopsy diagnosis, and AMC-EAC-031M (031M), established from a pretreatment biopsy of a liver metastasis of esophageal adenocarcinoma.

Immunofluorescence was performed as previously described (30). For cell culture and viability assay, flow cytometry, limiting dilution assays, migration assay, quantitative RT-PCR, survival, gene set enrichment analysis, and gene correlations (SI Appendix, Supplementary Materials and Methods).

Cytokine Array. The growth factor array AAH-CYT-4000 (RayBiotech) was performed according to the manufacturer’s protocol using 081RF supernatant incubated for 3 d, with unconditioned supernatant as the control. Detection was carried out using a Fujifilm LAS4000, and spot intensity was quantified using Image J. Fold induction was calculated according to the manufacturer’s instructions; each value was controlled for the positive control spots on each membrane and the values obtained from the unconditioned culture medium. Western blot and immunofluorescence analyses were carried out using antibodies from the membrane. For mouse-derived CAFs, the Mouse Cytokine Array C2000 (RayBiotech) was used. For IL-6 measurements on cell cultures, see SI Appendix, Supplementary Materials and Methods.

Western Blot. Western blots were performed as previously described (54). Following transfer, membranes were cut to allow detection of multiple antigens, guided by prestained molecular weight markers. See SI Appendix, Fig. S7 for full membranes and cutting strategy. Dashed boxes indicate crops shown in Fig. 2I. Primary antibodies (listed in SI Appendix, Table S1) were incubated overnight at 4°C. Proteins were imaged using Lumi-Light plus Western blot substrate (Pierce, Thermo Scientific) on a Fujifilm LAS 4000 imager. In parallel to the ECL images, epi-illuminated photographs were captured to document membrane topology.

Organoid Cultures. Early-passage PDX-derived organoids (P1-10) were cultured in 24-well plates in drops of 50 μL Matrigel (Coming) and maintained in serum-free Advanced DMEMF12 ( Gibco), supplemented with N2 supplement (Invitrogen), 2 μM β-glutamine, 100 μM β-mercaptoethanol (Sigma), trace elements B and C (Fisher Scientific), 5 mM Hepes (Life Technologies), 2 μg/mL heparin (Sigma), 10 μg/mL insulin (Sigma), and 0.15% Δ-glucone (Sigma). For passaging, organoids and Matrigel were mechanically disrupted in unsupplemented Advanced DMEMF12 medium (wash medium). The organoids were washed and resuspended two times before passaging. Different culture conditions as indicated were maintained during the assay; chemoradiation was given before passaging as indicated in Fig. 4. Medium was refreshed twice a week.
Clonogenic Assay. The 0.078 and 0.031M organoids cultivated in either control medium, medium containing 25% 081RF supernatant, or 25% 081RF supernatant for 30 min with IL-6-neutralizing antibody were treated with paditaxel and carboplatin at the indicated concentrations and irradiated (7x 1 Gy). After 2 wk, the organoids were replated in six-well plates at a density of 2,000 cells/well. Colony formation was determined after 4 wk, using crystal violet. During the assay, the culture medium was refreshed twice a week according to the conditions stated above.

Serum Marker Analysis. Serum from patients diagnosed with EAC in the study was stored at −80 °C, as approved by the institute’s ethical committee (MEC 01/28808017.1042). Informed consent was obtained from all included patients, and blood was drawn before the start of neoadjuvant treatment according to the CROSS regimen. The human IL-6 and human ADAM12 ELISA (both R&D Systems Duoset) were performed according to the manufacturer’s procedures.

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