Animal Model

COLO205 (ATCC-CCL-222) and HT29 (ATCC-HTB-38) human colorectal adenocarcinoma cells were grown in complete RPMI1640 and DMEM medium, respectively, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum and 100 U/mL penicillin plus 100 mg/mL streptomycin (Life Technologies). Both cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2. After subcutaneous cell injection, tumor dimensions were measured daily using a digital caliper, with tumor volumes calculated according to the formula: (length x width^2)/2. Blood glucose levels were measured (in duplicate) before ^{18}F-FDG PET scans from a drop of blood from the tail vein using a blood glucose meter (One Touch Ultra 2, Lifescan). Mice were subjected to μSPECT/CT followed by μPET/CT imaging just before (baseline) and 24 hours after therapy for treatment-response assessment as depicted in Supplemental Figure 2.

Control Peptide Preparation

To confirm the binding specificity of ^{99m}Tc-duramycin, a 19-amino acid linear control peptide (American Peptide Corporation) in which the thioether-linked amino acids are replaced by alanines and arginines are substituted for lysines (Ala-Arg-Gln-Ala-Ala-Ala-Phe-Gly-Pro-Phe-Ala-Phe-Val-Ala-Asp-Gly-Asn-Ala-Arg) was obtained. The linear peptide was reacted with HYNIC-NHS ester in DMSO purified by HPLC and radiolabeled with ^{99m}Tc (^{99m}Tc-linear duramycin; Supplemental Fig. 1A) as previously described for ^{99m}Tc-duramycin (18). The mass spectrum of HYNIC-linear duramycin control peptide showed the desired peptide mass. MS (ESI+) Calcd. $[M+H]^+$: 2112; found: 2112 (Supplemental Fig. 1B).

Imaging Studies

All mice were fasted overnight (10 hours), after which they received an intravenous (i.v.) injection of ~37 MBq ^{99m}Tc-duramycin or ^{99m}Tc-linear duramycin via the lateral tail vein at baseline and, 24 hours after treatment. Static whole-body SPECT imaging was performed using a μSPECT/CT scanner (VECTor/CT, MILabs) equipped with 75 pinholes of 1 mm, followed by CT acquisition (45 kV and 615 μA) for attenuation correction and anatomical coregistration with SPECT images, as described before (*1*). Mice were anesthetized through isoflurane inhalation (5% for induction and 2% for maintenance) and kept at constant body temperature during the scans. SPECT images were reconstructed with ordered-subsets expectation maximization (10 iterations, 16 subsets) and 1.2 mm^3 voxel size, and smoothed with a three dimensional isotropic Gaussian filter of 1 mm for visualization purposes. A 20% energy window centered at 140 keV photopeak was used. Immediately after μSPECT/CT imaging, while still anesthetized, the mice received an injection of 18.5 MBq ^{18}F-FDG, via a tail vein catheter, followed by a 30 minutes uptake period. After this, CT imaging was performed (80 keV, 500 μA, 220° rotation with 120 rotation steps) for PET data attenuation correction and anatomical reference (Siemens Inveon μPET/CT scanner). Static whole-body ^{18}F-FDG μPET images were next acquired over 20 minutes. The CT acquisitions were analytically reconstructed using the Feldkamp algorithm to a 352 x 352 x 606 matrix with 0.223 mm voxels. The μPET data were reconstructed using 2 iterations with 16 subsets of the three-dimensional ordered-subset expectation maximization (OSEM3D) and 18 maximum a posteriori (MAP) iterations, including scatter and

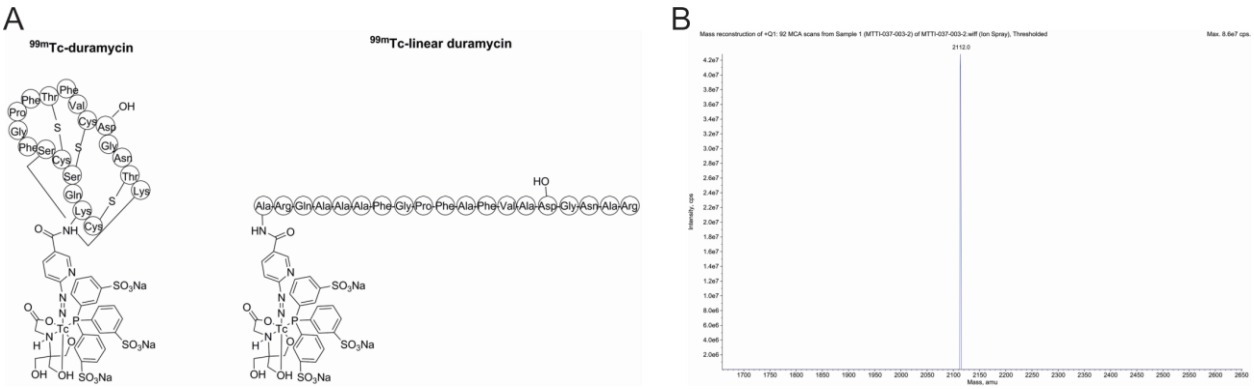
attenuation correction, resulting in a 128 x 128 x 159 matrix of 0.77 x 0.77 x 0.79 mm voxels (*2*).

Based on the size of the scanner's field of view (FOV), volumes of interest (VOIs) for the subcutaneous tumors were outlined on the CT images using PMOD v3.3 (PMOD Technologies). Radiotracer uptake was obtained from mean voxel intensity values within the delineated VOIs and then converted to Megabecquerels per milliliter (MBq/mL) using the calibration factor determined for the VECTor/CT μSPECT and Inveon μPET systems. The image VOI-derived percent injected dose per mL (%ID/mL) was calculated as [total radioactivity concentration in the VOI at the time of scan (kBq/mL)/total radioactivity injected (kBq) x 100].

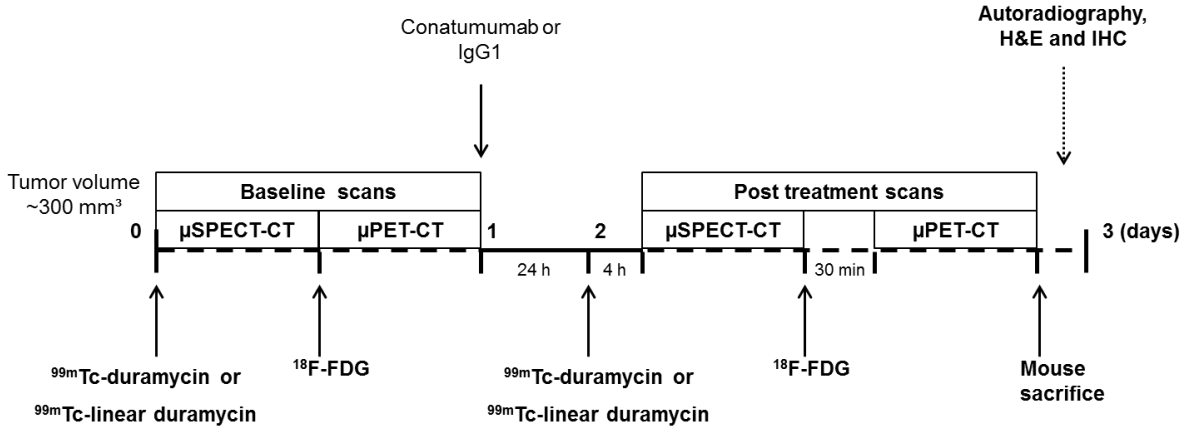
Immunohistochemistry

Tumors were fixed for 48 hours in 10% buffered formalin, and transferred to 70% ethanol until paraffin embedding. Immunohistochemistry for cleaved caspase-3 (CC3, rabbit monoclonal anti-CC3, 1:400 dilution, Cell Signaling Technology) and TdT-mediated dUTP nick-end labeling (TUNEL) assay (Promega) were carried out according to the manufacturer's instructions. The sections were counterstained with Ehrlich's hematoxylin. Tumors were also stained using standard hematoxylin and eosin (H&E) protocol and evaluated by a pathologist (S. Kumar-Singh). Light microscopic images were grabbed using a AxioScope A1 microscope (Zeiss) coupled with an Olympus UC30 color camera. Quantification of apoptosis was performed by calculating the percentage of CC3- and TUNEL-stained area across three non-sequential whole-tumor sections using IHC profiler plug-in for ImageJ v1.47, as previously described (*3*). Seven

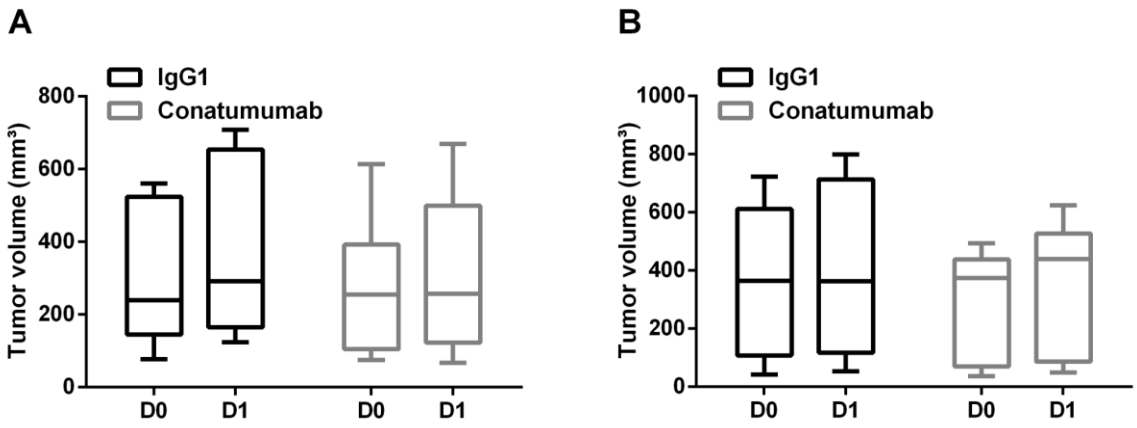
to nine tumors were evaluated per treatment (conatumumab- or IgG1-treated) and tumor type (COLO205 or HT29). Mean percentage of positive stained area per tumor was used to calculate differences between groups. Apoptosis levels were correlated to the corresponding *ex vivo* radiotracer uptake in the tumor.



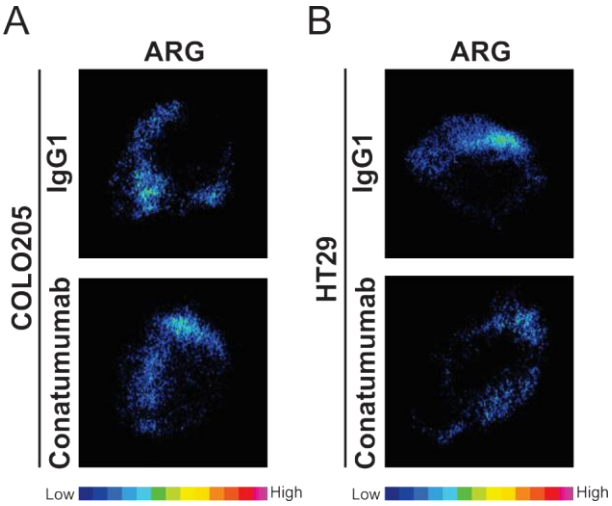
Supplemental Figure 1. Chemical structure for ^{99m}Tc-duramycin and ^{99m}Tc-linear duramycin (A). Mass spectrum of HYNIC-linear duramycin control peptide (B) showing the desired peptide mass. MS (ESI+) Calcd. $[M+H]^+$: 2112; found: 2112.



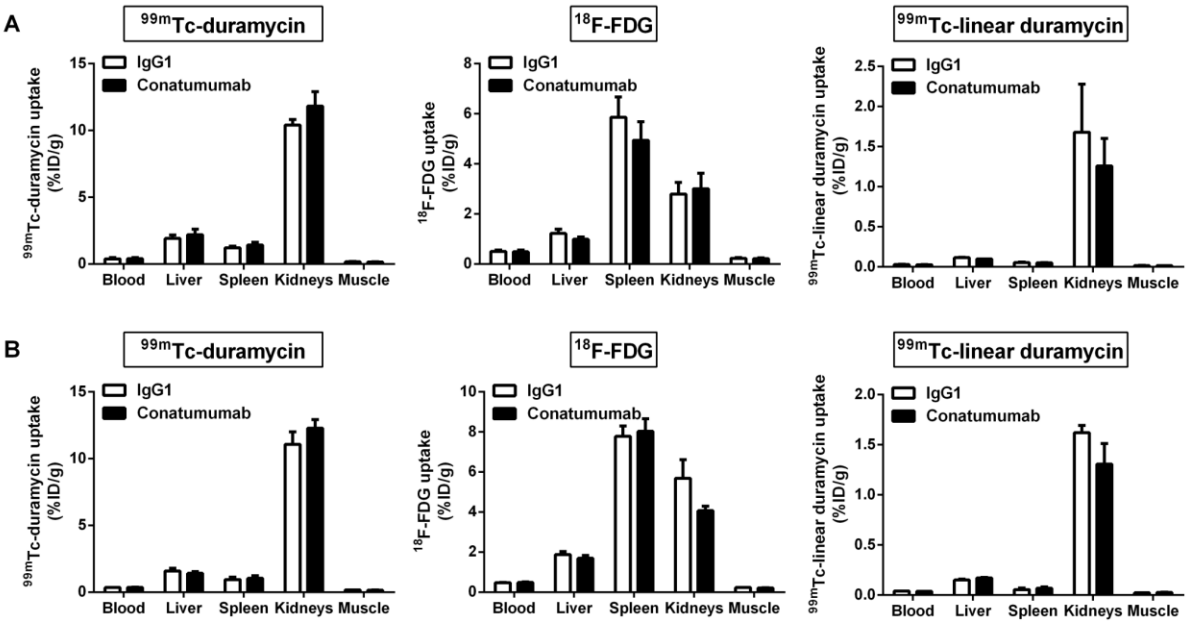
Supplemental Figure 2. Schematic representation of the experimental protocol using ^{99m}Tc-duramycin or ^{99m}Tc-linear duramycin SPECT/CT and ^{18}F-FDG PET/CT imaging in tumor-bearing mice treated with conatumumab or IgG1 control. H&E = hematoxylin and eosin; IHC = immunohistochemistry.



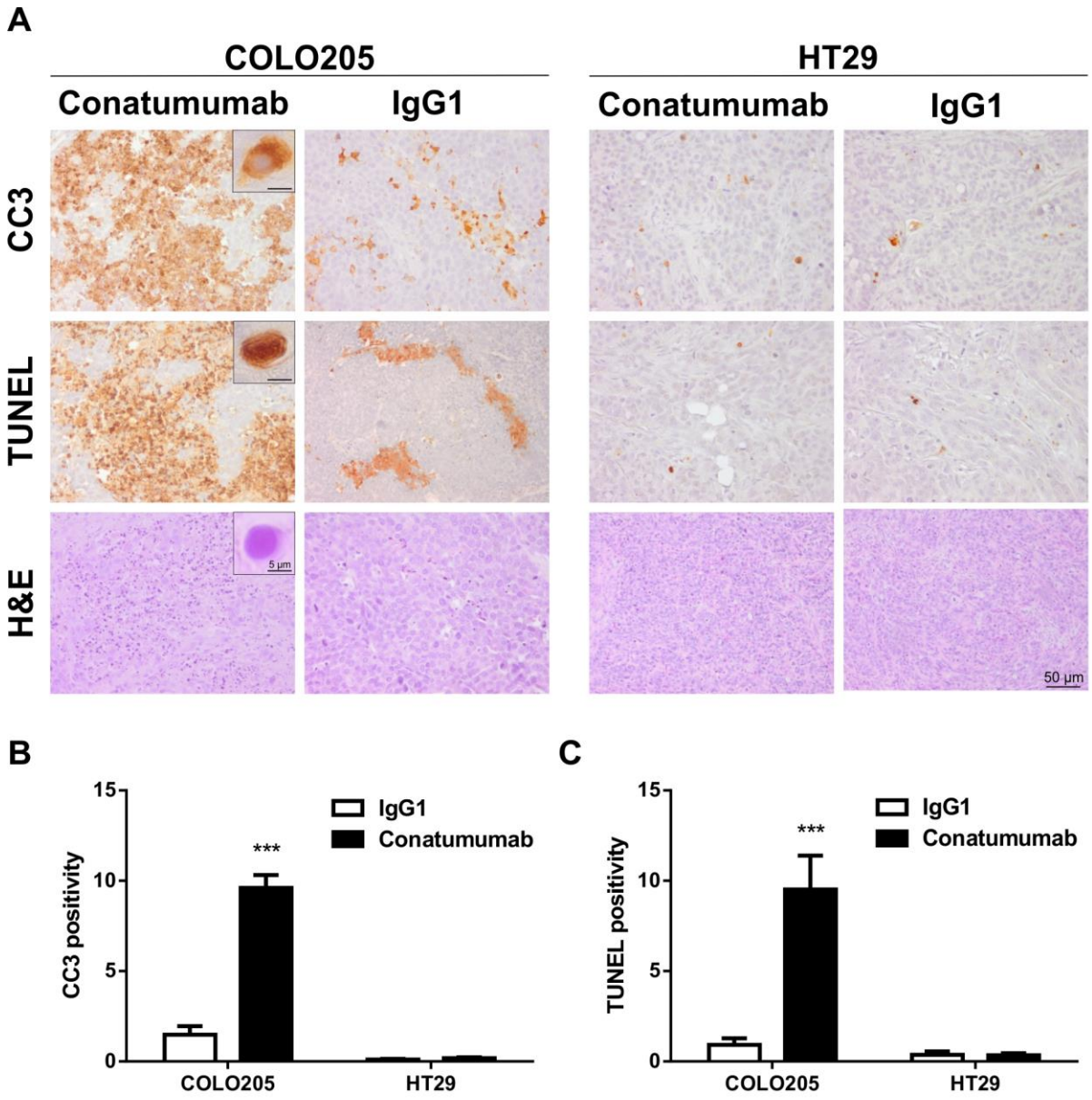
Supplemental Figure 3. Tumor volumes recorded by caliper measurements of COLO205 (A) and HT29 (B) tumor-bearing mice at baseline and after IgG1 or conatumumab treatment.



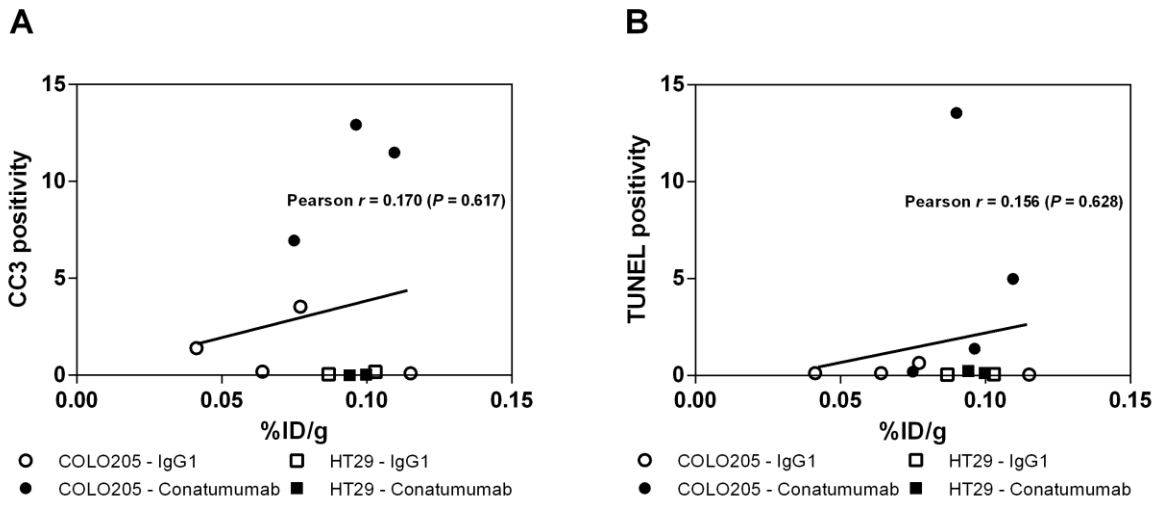
Supplemental Figure 4. Representative pseudo-color autoradiography (ARG) images of COLO205 (A) and HT29 (B) whole tumor slices from mice injected with ^{99m}Tc-linear duramycin after IgG1 or conatumumab treatment.



Supplemental Figure 5. *Ex vivo* ^{99m}Tc-duramycin, ^{18}F-FDG and ^{99m}Tc-linear duramycin organ biodistribution (%ID/g) of COLO205 (A) and HT29 xenografts (B) 24 hours after IgG1 or conatumumab treatment.



Supplemental Figure 6. Representative microscopic images of adjacent COLO205 and HT29 tumor slices stained with CC3, TUNEL and H&E acquired at x20 and x63 (inset) magnification. Cells in brown were positive for cleaved caspase-3 (CC3) and TUNEL staining (A). Densitometric analysis of CC3 (B) and TUNEL (C) staining. ***, $P < 0.001$, significantly different from IgG1.



Supplemental Figure 7. Correlation analysis of radiotracer uptake to histological measurement of tumor cell death. CC3 (A) and TUNEL staining (A) were compared with tumor-associated ^{99m}Tc-linear duramycin radioactivity measured *ex vivo* in the tumors (%ID/g).

Supplemental Table 1. Blood glucose levels in COLO205 and HT29 xenografts at baseline and after therapy

Scan	COLO205		HT29	
	IgG1	Conatumumab	IgG1	Conatumumab
Baseline (mg/dL)	68.6 ± 9.4	82.5 ± 2.3	58.2 ± 9.1	62.5 ± 7.4
Post treatment (mg/dL)	87 ± 6.5	77.4 ± 7.3	67.8 ± 13.5	52.8 ± 3.3

Data are mean ± SEM

Supplemental Table 2. Uptake of ^{18}F-FDG in COLO205 and HT29 xenografts at 24 hours post treatment and tumor-to-background ratios

Parameter	COLO205		HT29	
	IgG1	Conatumumab	IgG1	Conatumumab
Blood uptake (%ID/g)	0.49 ± 0.06	0.48 ± 0.07	0.47 ± 0.01	0.48 ± 0.03
Muscle uptake (%ID/g)	0.22 ± 0.03	0.21 ± 0.03	0.23 ± 0.01	0.20 ± 0.02
T/B ratio	4.95 ± 0.81	6.02 ± 1.09	3.74 ± 0.21	4.09 ± 0.55
T/M ratio	11.60 ± 1.47	13.71 ± 2.65	7.74 ± 0.34	8.47 ± 1.01

Data are mean ± SEM

Supplemental Table 3. Uptake of ^{99m}Tc-linear duramycin in COLO205 and HT29 xenografts and tumor-to-background ratios

Parameter	COLO205		HT29	
	IgG1	Conatumumab	IgG1	Conatumumab
Blood uptake (%ID/g)	0.028 ± 0.004	0.026 ± 0.001	0.038 ± 0.001	0.035 ± 0.002
Muscle uptake (%ID/g)	0.014 ± 0.002	0.013 ± 0.001	0.021 ± 0.001	0.025 ± 0.002
T/B ratio	2.91 ± 0.57	3.57 ± 0.41	2.46 ±.18	2.74 ± 0.24
T/M ratio	5.31 ± 0.70	7.02 ± 0.43	4.62 ± 0.73	3.83 ± 0.14

Data are mean ± SEM

References

1. Elvas F, Vangestel C, Pak K, et al. Early Prediction of Tumor Response to Treatment: Preclinical Validation of 99mTc-Duramycin. *J Nucl Med.* 2016;57:805-811.

2. Deleye S, Heylen M, Deiteren A, et al. Continuous flushing of the bladder in rodents reduces artifacts and improves quantification in molecular imaging. *Mol Imaging.* 2014;13.

3. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One.* 2014;9:e96801.