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- coding region into the Not I-Xba I sites of the expression vector pcDNA3.1 (Invitrogen) and generated pcDNA3.1-AIB1.
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  11. We obtained established breast cancer cell lines from the American Type Culture Collection (ATCC) (BT474, MCF-7, T-47D, MDA-MB-361, MDA-MB-468, BT-20, MDA-MB-436, and MDA-MB-453), the Arizona Cancer Center (UACC-812), and the National Cancer Institute (ZR75-1). We obtained ovarian cancer cell line BG-1 from Jeff Boyd, University of Pennsylvania. For Northern analysis, we obtained normal human mammary gland total RNA pooled from six individuals between 16 and 35 years old from Clontech. For FISH analysis, interphase nuclei were fixed in methanol and acetic acid (3:1) and dropped onto microscope slides.
  12. Data not shown.
  13. The BioPrime DNA labeling system (Gibco BRL) was used to label genomic clones containing either AIB1 (3) or the 20q13 amplicon (RMC20C001) (14) with Spectrum Orange deoxyuridine triphosphate (dUTP) (Vysis). We used 20q11 and 20p probes (University of California, Berkeley, Resource for Molecular Cytogenetics RMC20P037 and RMC20P039) as reference probes for two-color FISH analysis after labeling with biotin-16-dUTP (BMB) by nick-translation. FISH followed the method of Pinkel *et al.* (19) with minor modifications. Fluorescent images were captured with a Zeiss axiophot microscope equipped with a charge-coupled device camera and IP Lab Spectrum software (Signal Analytics). FISH analysis of uncultured breast cancer samples was performed as described (14) from either disaggregated nuclei or sections of ethanol-fixed tumors. Tumors were categorized into three groups according to AIB1 copy number: low (fewer than four copies per cell or no increased relative copy number), moderate (four to six copies per cell or 1.5- to 3-fold relative copy number), and high (more than six copies per cell or >3-fold relative copy number). Only high copy number increases were taken as evidence for gene amplification. In 105 unselected cases, 10 were scored high, 13 were moderate, and 82 were low. ER status was determined by immunohistochemistry for 83 of these specimens including 9 of the 10 cases with AIB1 amplification. Of these nine specimens, four were ER positive and five were ER negative.
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  15. AIB1 mRNA in situ expression was determined by using three cDNA fragments (covering nucleotides 1733 to 2579, 3072 to 3580, and 3533 to 4120 of the AIB1 cDNA) labeled with deoxyadenosine [<sup>32</sup>P]-triphosphate by PCR. We hybridized ethanol-fixed sections from 75 primary breast tumors and six adjacent normal breast tissues as described (20). We knew the ER status for 66 of these samples. AIB1 was highly expressed in 24 of 44 (55%) of the ER-positive cases and in 8 of 22 (36%) of the ER-negative cases.
  16. CV-1 cells (ATCC) were grown and maintained in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal bovine serum. We plated cells into six-well culture dishes at  $1.0 \times 10^5$  cells per well and allowed them to grow overnight. Transfections were done with calcium phosphate coprecipitation (Clontech) according to the manufacturer's protocol. Transfected DNA included 5 ng of pRL-CMV internal control plasmid, 1.0  $\mu$ g of ER reporter, 250 ng of pHEGO-hyg ER expression vector, the indicated amount of pcDNA3.1-AIB1, or an equivalent amount of pcDNA3.1 empty vector, and salmon sperm DNA to a total of 7  $\mu$ g of DNA per dish. After transfection, we incubated cells in the absence or presence of 10 nM 17 $\beta$ -estradiol or 100 nM 4-OHT. Cell lysates were harvested and assayed 48 hours after transfection. We determined reporter activities with the dual-luciferase reporter assay system (Promega) and the results are expressed in relative luminescence units (RLU) (luciferase/*Renilla* luciferase). We obtained pRL-CMV and pGL3-promoter from Promega and pHEGO-hyg from ATCC. ER reporter pGL3.luc.3ERE, which contains three tandem copies of the ERE upstream from the simian virus 40 promoter driving the luciferase gene, was the kind gift of Fern Murdoch, Uniformed Services University of the Health Sciences.
  17. We constructed GST fusion proteins by generating PCR fragments of AIB1 encoding amino acids 1 to 194 and amino acids 605 to 1294 inserted into pGEX 6P-2 (Pharmacia). GST pull-down analysis was done as described by Le Douarin (21) with the following modifications: 6.7  $\mu$ g of ER (Panvera) was preincubated with or without 2  $\mu$ M estradiol. Separately, we preincubated 10  $\mu$ g of either GST, GST-AIB.N1 (containing amino acids 1 to 194), or GST-AIB.T1 (containing amino acids 605 to 1294) with preequilibrated glutathione-Sepharose (Pharmacia) and washed it with binding buffer. ER with or without estradiol mixture was incubated with GST fusion protein-glutathione-Sepharose mixture in binding buffer containing 0.1% bovine serum albumin for 1 hour at room temperature. Glutathione-Sepharose beads were washed five times in binding buffer, and bound proteins were eluted in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and separated by SDS-PAGE. Separated proteins were transferred to nylon membranes, incubated sequentially with monoclonal antibody H-151 to ER (Stress-Gen) and horseradish peroxidase containing goat anti-mouse immunoglobulin G Fc (Jackson Immuno-research), and detected by chemiluminescence.
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  23. We obtained cDNA clones from Research Genetics [TIF2 (clone 132364, GenBank accession number R25318), SRC-1 (clone 418064, GenBank accession number W90426)], ATCC (pHEGO-hyg, ATCC number 79995), or Clontech ( $\beta$ -actin). The AIB1 probe was a 2.2-kb Not I-Sac I fragment of pCMVSPORT-B11.
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  25. The authors gratefully acknowledge the technical assistance of Nasser Parsa and Robin Veldman, Darryl Leja for preparation of the illustrations, and Roger Meisfeld for helpful discussions.

22 April 1997; accepted 14 July 1997

## Pain Affect Encoded in Human Anterior Cingulate But Not Somatosensory Cortex

Pierre Rainville, Gary H. Duncan, Donald D. Price, Benoît Carrier, M. Catherine Bushnell\*

Recent evidence demonstrating multiple regions of human cerebral cortex activated by pain has prompted speculation about their individual contributions to this complex experience. To differentiate cortical areas involved in pain affect, hypnotic suggestions were used to alter selectively the unpleasantness of noxious stimuli, without changing the perceived intensity. Positron emission tomography revealed significant changes in pain-evoked activity within anterior cingulate cortex, consistent with the encoding of perceived unpleasantness, whereas primary somatosensory cortex activation was unaltered. These findings provide direct experimental evidence in humans linking frontal-lobe limbic activity with pain affect, as originally suggested by early clinical lesion studies.

**A**ffective aspects of pain, such as perceived unpleasantness, have been classically considered to be distinct from the simple sensory dimensions of pain, which include the perception of location, quality, and intensity of noxious stimulation (1). Largely on the basis of indirect evidence, separate neuronal pathways have been postulated to underlie these different aspects of the pain experience (2). For example, involvement of frontal lobe regions, particularly the anterior cingulate cortex (ACC), in pain affect is suggested by clinical reports that patients with frontal lobotomies or cingulotomies sometimes still feel pain but report it as less distressing or bothersome (3). On the other hand, primary and secondary somatosensory cortices (SI and SII) have been considered plausible candidates for the processing of sensory-discriminative aspects of pain, on the basis of their anatomical connections to subcortical and spinal re-

gions, which encode discriminative properties of somatosensory stimuli (4). Recent neuroimaging studies in humans documented pain-related activation in limbic sites, such as ACC and rostral insula (IC), and in the primary sensory regions SI and SII (5). In addition, anatomical and electrophysiological data show that these regions receive direct nociceptive input in the monkey (6). However, the extent to which these different cortical structures contribute to specific dimensions of the human pain experience is largely unknown and untested.

In the present study we used hypnosis as a cognitive tool to reveal possible cerebral mechanisms of pain affect in normal human volunteers. A perceptual dissociation of sensory and affective aspects of the pain experience was achieved with hypnotic suggestions to both increase and decrease pain unpleasantness, without changing the perceived intensity of the pain sensations (7).

Cerebral cortical activity related to this perceptual dissociation was measured by positron emission tomography (PET) (8).

PET scans were conducted during conditions of alert control, hypnosis control, and hypnotic suggestion for increased unpleasantness (↑UNP) or decreased unpleasantness (↓UNP) (9). During each scan tonic stimuli were presented to the left hand by passive immersion in “neutral” (35°C) or “painfully hot” (47°C) water (10). After each scan, the perceived intensity and unpleasantness of the stimulation were rated by the participant (11).

Regional cerebral blood flow (rCBF) was measured with three-dimensional high-resolution PET after H<sub>2</sub><sup>15</sup>O bolus injection (12). Each participant also received a high-resolution magnetic resonance imaging (MRI) anatomical brain scan that was used for alignment and transformation of PET volumes into the Talairach coordinate system (13). To obtain volumes of pain-related changes in rCBF for each participant, we subtracted normalized PET data recorded during the “neutral” condition from those of the “painfully hot” condition. Resulting volumes of pain-related changes in rCBF were averaged across sessions, and statistical activation maps were derived on the basis of the methods of Worsley *et al.* (14). Directed searches of rCBF increases were conducted on right (contralateral to stimulus) SI, SII, ACC, and IC to confirm pain-related activation of these structures and to test the hypothesis that changes in pain unpleasantness modulate activity only within limbic regions thought to be involved in affective processes. The threshold for statistical significance was corrected for multiple comparisons (15).

Results of “painfully hot” versus “neutral” subtractions from scans taken during the alert control condition support previous findings of significant pain-related activa-

tions in SI, SII, IC, and ACC (Table 1). After hypnotic induction, but before suggestions of increased or decreased unpleasantness, painful heat again activated these four cortical areas (Table 1), indicating little influence of hypnotic induction itself on pain-related activation. Similarly, the hypnotic induction had no significant effect on psychophysical ratings of either pain intensity or unpleasantness (alert control compared with hypnosis control: intensity, 77.6 ± 14.7 and 75.0 ± 14.0, and unpleasantness, 61.4 ± 28.8 and 54.8 ± 25.8, respectively).

Hypnotic suggestions for increased or decreased unpleasantness, on the other hand, altered both the perception of pain affect and the activation within some but not all of these pain-related cortical regions. A comparison of rCBF changes between hypnotic suggestion and hypnosis control conditions revealed significant pain-related activations in SI, ACC, and IC, during both ↑UNP and ↓UNP conditions. Within the vicinity of SII, however, no significant pain-evoked activity was observed in either the ↑UNP or ↓UNP conditions (Table 1). One possible explanation for this absence of pain-evoked activity in SII is that the mental effort or attention demanded by these suggestions may suppress such activation. Alternatively, there may have been an habituation of SII activity with

repeated stimulation.

The effectiveness of hypnotic suggestions in selectively altering pain affect is demonstrated by the significant difference observed only in the participants’ ratings of unpleasantness during the ↑UNP and ↓UNP conditions [unpleasantness: 81.4 ± 14.6 and 45.0 ± 25.8, respectively, analysis of variance (ANOVA) *P* < 0.001; intensity: 78.0 ± 14.6 and 71.2 ± 18.2, respectively, statistically not significant]. In parallel with this modulation in pain affect, direct volume-of-interest (VOI) comparisons (16) of the three pain sites activated during the hypnotic suggestion conditions revealed significantly greater activation during ↑UNP scans, compared with that observed during ↓UNP, only in ACC (*P* < 0.02; see Fig. 1). In SI, pain-related rCBF was actually lower (nonsignificantly) in the ↑UNP condition than in the ↓UNP condition, indicating no tendency for increased activation in this area related to increased unpleasantness.

To test the strength of the relation between pain affect and activation within the ACC, we did regression analyses of unpleasantness ratings and rCBF levels across all participants and all scans taken during the hypnotic suggestion condition for each pain activation site. After removing effects due to interperson variability, perceived intensity, and scan session, the residual variance in rCBF [analysis of covariance (ANCOVA)] demonstrates that only activation levels

**Table 1.** Pain-related activation sites within SI, SII, ACC, and IC. Coordinates are given in Talairach space (13); Lateral, anterior, and superior are relative to midline, anterior commissure, and commissural line, respectively (positive values are right, anterior, and superior). A *t* statistic of 2.55 is equivalent to *P* = 0.05 (15).

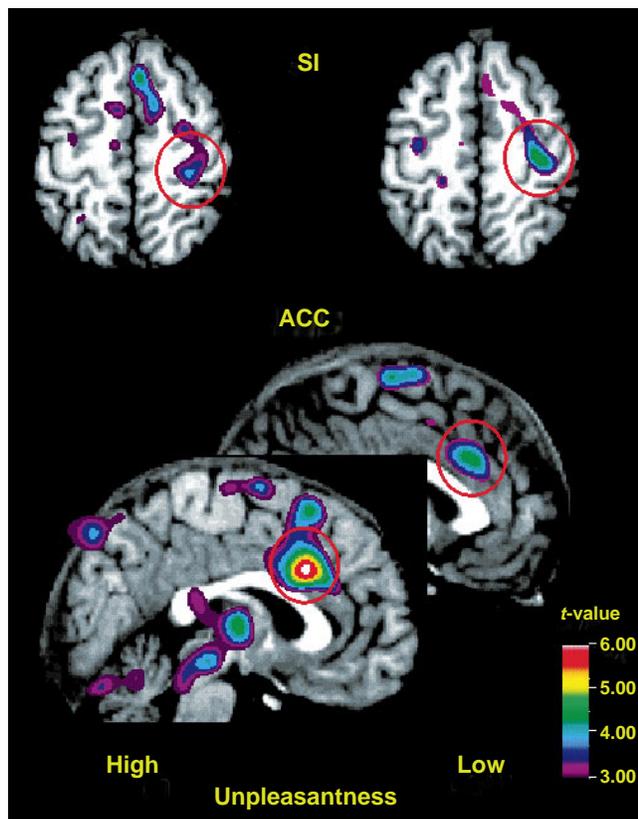
Region	Stereotaxic coordinates (mm)			<i>t</i>
	Lateral	Anterior	Superior	
	<i>Alert control</i>			
SI	+42	-18	+40	3.01
SII	+44	-23	+20	4.18
ACC	+1	+5	+36	4.04
IC	+42	-6	+12	3.61
	<i>Hypnosis control</i>			
SI	+39	-21	+59	3.69
SII	+40	-18	+20	2.65
ACC	-8	-4	+34	3.86
	+7	+18	+32	2.52*
	+16	+18	+36	3.34
IC	+31	+10	+12	3.77
	+35	+2	+1	4.16
	<i>Increased unpleasantness (↑UNP)</i>			
SI	+31	-28	+57	3.84
SII	No peak with <i>t</i> > 2.50			
ACC	+3	+20	+30	6.11
IC	+34	+22	0	4.50
	<i>Decreased unpleasantness (↓UNP)</i>			
SI	+34	-19	+56	4.61
SII	No peak with <i>t</i> > 2.50			
ACC	-1	+25	+29	4.42
	+13	+18	+36	3.64
IC	+38	+8	+3	4.66

\**P* = 0.053

P. Rainville, Département de Psychologie and Centre de Recherche en Sciences Neurologiques, Université de Montréal, Montréal, Québec, Canada H3C 3J7, and McConnell Brain Imaging Center, Montreal Neurological Institute, Montréal, Québec, Canada H3A 2B4.  
 G. H. Duncan, Département de Stomatologie, Faculté de Médecine Dentaire, and Centre de Recherche en Sciences Neurologiques, Université de Montréal, Montréal, Québec, Canada H3C 3J7, and McConnell Brain Imaging Center, Montreal Neurological Institute, Montréal, Québec, Canada.  
 D. D. Price, Department of Anesthesiology, Medical College of Virginia, Richmond, VA 23298, USA.  
 B. Carrier, Département de Stomatologie, Faculté de Médecine Dentaire, Université de Montréal, Montréal, Québec, Canada H3C 3J7.  
 M. C. Bushnell, Centre de Recherche en Sciences Neurologiques, Université de Montréal, Montréal, Québec, Canada H3C 3J7, McConnell Brain Imaging Center, Montreal Neurological Institute, Montréal, Québec, Canada, and Department of Anesthesiology, McGill University, Montréal, Québec, Canada H3A 1A1.

\*To whom correspondence should be addressed. E-mail: bushnellc@medcor.mcgill.ca

**Fig. 1.** Changes in pain-related activity associated with hypnotic suggestions of high and low unpleasantness (left and right images, respectively) are revealed by subtracting PET data recorded during the neutral/hypnosis control condition from those of the painfully hot/↑UNP and painfully hot/↓UNP conditions. PET data, averaged across 11 experimental sessions, are illustrated against an MRI from one person; horizontal and sagittal slices through SI and ACC, respectively, are centered at the activation peaks observed during the relevant suggestion conditions; red circles indicate the location and size of VOIs used to analyze activation levels across the two conditions (16).



within the ACC (Fig. 2) are consistent with the encoding of the perceived unpleasantness of these noxious stimuli (ACC: Pearson's correlation coefficient,  $r = 0.419$ ,  $P = 0.005$ ; IC:  $r = 0.245$ ,  $P = 0.134$ ; SI:  $r = -0.224$ ,  $P = 0.149$ ).

These results demonstrate a modulation of pain-related activity in ACC that closely parallels a selective change in the perceived unpleasantness of painful stimuli. The absence of changes in the sensory component of pain perception and the lack of similar modulation within other pain-related cortical structures argue for a significant involvement of the ACC in the affective component of pain. Such findings support earlier proposals that the anterior cingulate gyrus is integrally involved in pain and emotions (3, 5, 6), but our findings go beyond these general ideas by providing direct evidence of a specific encoding of pain unpleasantness in the ACC.

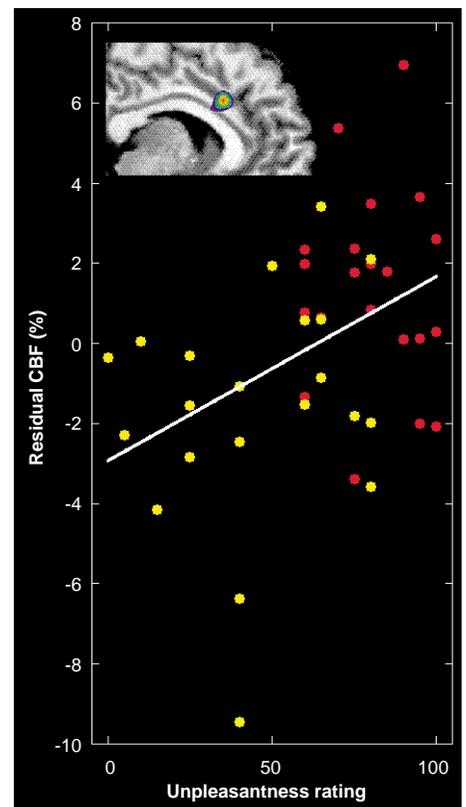
We propose that pain-related activation in ACC reflects a nociceptive input from a highly modifiable pain pathway (17), and that the level of pain-evoked ACC activation is determinant in the individual's emotional and behavioral reactions to pain. The proximity of the nociceptive, motor, and attentional regions of ACC (18) suggests possible local interconnections that might allow the output of the ACC pain area to command immediate behavioral reactions.

Similarly, the ACC pain area might participate in the substantial interconnections between the ACC and the "fight or flight" regions of the midbrain periaqueductal gray matter (19).

The anatomical connections between ACC, IC, SI, and SII (20) suggest that these regions do not function independently in encoding different aspects of pain but are highly interactive. Such interactions are reflected in the experiences of pain itself. For example, pain intensity, location, and quality (sensory features) are major factors in determining unpleasantness (21). Nevertheless, despite these associations, there appears to be at least a partial segregation of function between pain affect and sensation, with ACC activity possibly reflecting the emotional experience that provokes our reactions to pain.

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**Fig. 2.** Activation levels (as measured by residual rCBF) observed within the ACC during high (red) and low (yellow) unpleasantness conditions are significantly correlated with ratings of pain unpleasantness (ANCOVA:  $r = 0.42$ ,  $P = 0.005$ ). The coordinates of this  $r$ -value peak (inset: sagittal section of PET regression volume—lateral, +7; anterior, +20; superior, +29) lie precisely within the region of ACC that was activated in both ↑UNP and ↓UNP conditions (see Fig. 1 and Table 1). Line shows linear best fit.

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- years in age, who displayed moderate to high hypnotic suggestibility (Stanford Suggestibility Scale Form A) and robust modulation of pain unpleasantness were chosen for the PET study. Experimental sessions (12 scans) were administered once to each of five participants and twice to three other participants. Because of possible residual effects of hypnotic suggestions, the alert control conditions were always presented first, followed by hypnotic control (without suggestions of altered perception), and finally suggestions for increased ( $\uparrow$ UNP) or decreased ( $\downarrow$ UNP) unpleasantness. "Neutral" (35°C) and "painfully hot" (46.5° to 47.5°C) stimuli were counterbalanced across individuals within the alert and hypnotic control states, as were the blocks of two  $\uparrow$ UNP and two  $\downarrow$ UNP scans.
10. This 75-s hand immersion stimulus was chosen on the basis of our previous findings that tonic pain has a stronger affective component than phasic pain [P. Rainville, J. S. Feine, M. C. Bushnell, G. H. Duncan, *Somatosens. Mot. Res.* **9**, 265 (1992)]. Temperatures were adjusted individually to obtain pain ratings of 40 to 80 on a scale of 0 to 100 [see (17)]; resulting temperatures ranged from 46.5° to 47.5°C.
  11. Participants rated pain intensity and unpleasantness using separate numerical scales of 0 to 100. The intensity scale endpoints were "no burning, pricking, stinging sensation," the most frequently chosen words describing the sensory aspect of heat pain in an independent study [C. Morin, L. TenBokum, M. C. Bushnell, *Soc. Neurosci. Abstr.* **20**, 127 (1994)], and "extremely intense sensation." The unpleasantness scale endpoints were "not at all unpleasant" and "extremely unpleasant." To avoid ceiling effects, we instructed participants that responses could surpass 100 if larger values were needed to describe sensations relative to those previously rated.
  12. PET data (63 slices) were acquired with a Siemens ECAT HR+ camera. Participants lay immobile in the scanner, eyes closed, with inserted earphones connected to a microphone through which they received instructions or hypnotic suggestions before each scan. Stimulus onset was simultaneous with bolus injection (10 mCu of  $H_2^{15}O$ , half-life of 123 s, without arterial blood sampling) to synchronize the increase in pain sensation with brain uptake of  $H_2^{15}O$ . Scans began 15 s after the injection, and data were collected in two sequential frames of 40 and 20 s (data presented are derived from the 40-s frame, which yielded the better signal-to-noise ratio). Scans were separated by 12 to 15 min to allow tracer decay to background levels.
  13. MRI scans (160 contiguous 1-mm-thick slices) were acquired on a Philips 1.5T Gyroscan system. Each participant's PET and MRI volumes were transformed into the Talairach coordinate system [J. Talairach and P. Tournoux, *Co-Planar Stereotaxic Atlas of the Human Brain* (Thieme, New York, 1988)] by using the automated methods of D. L. Collins, P. Neelin, T. M. Peters, and A. C. Evans, [*J. Comput. Assisted Tomogr.* **18**, 192 (1994)]. PET and MRI volumes were resampled to obtain voxels of 1.34 mm by 1.72 mm by 1.50 mm in the x, y, and z planes, respectively.
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  15. With four target foci, the search volume of 4 resels yields a threshold for statistical significance of  $t = 2.55$  ( $P < 0.05$ , one-tailed  $t$ -test corrected for multiple comparisons).
  16. VOIs were centered independently at the point of maximum pain-related increase in rCBF within each of the three pain sites identified in the two comparison conditions. This procedure ensured that the maximum activation observed in  $\uparrow$ UNP was directly compared with the corresponding point of maximum activation obtained in  $\downarrow$ UNP. To further verify the robustness of these comparisons, we tested different VOI radii (from 10 to 20 mm) for each structure, with similar results for all values.
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14 April 1997; accepted 24 June 1997

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