stimulation of myogenic differentiation by NLS-Smad4(ΔMH1); this mutant Smad4 lacking DNA-binding activity may quench the transcriptional activity of the complex via the MH2 domain. It also appeared that a component of the Smad4 complex, interacting through the MH2 domain, is critical for inhibition of myogenic differentiation in response to BMPs. In the present study, we identified E4F1 as one of the components of the Smad4 complex in the nucleus, interacting through the MH2 domain. E4F1 is a zinc finger DNA-binding protein, identified as a cellular target of viral oncoproteins and shown to regulate the cell cycle (40–42). Our findings indicated that overexpression of E4F1 inhibited myogenic differentiation cooperatively with Smad4. Moreover, RNAi knockdown of E4F1 prevented the inhibition of myogenic differentiation by BMP signaling. Although E4F1 was recently shown to act as a ubiquitin E3 ligase of p53 (43), our findings indicated that deletion of the ubiquitin E3 ligase domain from E4F1 still allowed inhibition of myogenic differentiation. However, all of the zinc finger structures of E4F1 seemed to be important for this inhibitory activity. Taken together, these findings suggest that Smad4, which undergoes nuclear translocation in response to BMP stimulation, may interact with E4F1 in the nucleus to suppress myogenic differentiation as a transcription factor, independent of its ubiquitin E3 ligase activity. Recently, it was reported that Smad4 regulates the processing of pri-microRNA into mature microRNA in response to BMP-2 treatment (44). The direct target gene(s) of the complex still needs to be identified. It is interesting to note that loss-of-function mutations of p53 and Smad4 were identified in some tumors, suggesting that mutations in the Smad4-E4F1-p53 axis might play a role in tumorigenesis (45, 46).

We found that E4F1 stimulated the expression of *Id1–3* in the presence of Smad4. Id proteins inhibit myogenesis and are targets of BMP signaling. Recently, insufficient skeletal muscle repair was reported in $Id1^{+/-}Id3^{-/-}$ mice after muscle injury (47). BMP signaling may also up-regulate *Id* expression in healing muscle tissue (47). Because expression of *Ids* leads to cell cycle progression, the E4F1-induced Ids may suppress myogenic differentiation and maintain myoblast proliferation. Further studies are needed to elucidate the physiological roles of Smads and E4F1 in muscle development and regeneration *in vivo*.

In the present study, we obtained an unexpected finding related to Smads. BMP-induced osteoblastic differentiation was not completely blocked in the *Smad4*-deleted MEFs. There are some possible explanations for this finding: 1) undetectable levels of Smad4 still remained in the MEFs expressing Cre recombinase, 2) an alternative pathway, including a novel Co-Smad, transduced BMP signaling, or 3) Smad4 is not essential for the osteoblastic differentiation induced by BMPs. Recently, evidence has been presented that bone and cartilage tissues were formed during development in the absence of functional Smad4 in mice, although such mice exhibited abnormalities (48). Deletion of *Smad4* in mouse mature osteoblasts using a Cre-loxP system significantly reduced bone volume and osteoblast function *in vivo*, but they still had bone tissues and osteoblasts (48). Further study will be required to elucidate the roles of Smad4 in bone metabolism.

In conclusion, we found that the Smad-dependent pathway regulates both the inhibition of myogenic differentiation and the induction of osteoblastic differentiation induced by BMPs. The introduction of negative charges at the carboxyl terminus of Smad1 may play an important role in the induction of osteoblast differentiation in response to BMPs. In contrast, nuclear Smad4, rather than R-Smad, and E4F1, a novel partner of nuclear Smad4, are responsible for the inhibition of myogenic differentiation by BMPs.

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REFERENCES

1. Katagiri, T., Suda, T., and Miyazono, K. (2008) *The Bone Morphogenetic Proteins*, pp. 121–149, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Urist, M. R. (1965) *Science* **150,** 893–899
3. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) *Science* **242,** 1528–1534
4. Wozney, J. M., and Rosen, V. (1998) *Clin. Orthop. Relat. Res.* **346,** 26–37
5. Sampath, T. K., Muthukumaran, N., and Reddi, A. H. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7109–7113
6. Salminen, A., Braun, T., Buchberger, A., Jürs, S., Winter, B., and Arnold, H. H. (1991) *J. Cell Biol.* **115,** 905–917
7. Yoshida, S., Fujisawa-Sehara, A., Taki, T., Arai, K., and Nabeshima, Y. (1996) *J. Cell Biol.* **132,** 181–193
8. Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1994) *J. Cell Biol.* **127,** 1755–1766
9. Massagué, J., Cheifetz, S., Endo, T., and Nadal-Ginard, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8206–8210
10. Liu, D., Black, B. L., and Derynck, R. (2001) *Genes Dev.* **15,** 2950–2966
11. Miyazono, K., Maeda, S., and Imamura, T. (2005) *Cytokine Growth Factor Rev.* **16,** 251–263
12. Wan, M., and Cao, X. (2005) *Biochem. Biophys. Res. Commun.* **328,** 651–657
13. Katagiri, T., Imada, M., Yanai, T., Suda, T., Takahashi, N., and Kamijo, R. (2002) *Genes Cells* **7,** 949–960
14. Liu, C. J., Ding, B., Wang, H., and Lengyel, P. (2002) *Mol. Cell. Biol.* **22,** 2893–2905
15. López-Rovira, T., Chalaux, E., Massagué, J., Rosa, J. L., and Ventura, F. (2002) *J. Biol. Chem.* **277,** 3176–3185
16. Shore, E. M., Xu, M., Feldman, G. J., Fenstermacher, D. A., Cho, T. J., Choi, I. H., Connor, J. M., Delai, P., Glaser, D. L., LeMerrer, M., Morhart, R., Rogers, J. G., Smith, R., Triffitt, J. T., Urtizberea, J. A., Zasloff, M., Brown, M. A., and Kaplan, F. S. (2006) *Nat. Genet.* **38,** 525–527
17. Fukuda, T., Kohda, M., Kanomata, K., Nojima, J., Nakamura, A., Kamizono, J., Noguchi, Y., Iwakiri, K., Kondo, T., Kurose, J., Endo, K., Awakura, T., Fukushi, J., Nakashima, Y., Chiyonobu, T., Kawara, A., Nishida, Y., Wada, I., Akita, M., Komori, T., Nakayama, K., Nanba, A., Maruki, Y., Yoda, T., Tomoda, H., Yu, P. B., Shore, E. M., Kaplan, F. S., Miyazono, K., Matsuoka, M., Ikebuchi, K., Ohtake, A., Oda, H., Jimi, E., Owan, I., Okazaki, Y., and Katagiri, T. (2009) *J. Biol. Chem.* **284,** 7149–7156
18. Maruyama, Z., Yoshida, C. A., Furuichi, T., Amizuka, N., Ito, M.,

Fukuyama, R., Miyazaki, T., Kitaura, H., Nakamura, K., Fujita, T., Kanatani, N., Moriishi, T., Yamana, K., Liu, W., Kawaguchi, H., Nakamura, K., and Komori, T. (2007) *Dev. Dyn.* **236,** 1876–1890
19. Goldman, L. A., Cutrone, E. C., Kotenko, S. V., Krause, C. D., and Langer, J. A. (1996) *BioTechniques* **21,** 1013–1015
20. Katagiri, T., Akiyama, S., Namiki, M., Komaki, M., Yamaguchi, A., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1997) *Exp. Cell Res.* **230,** 342–351
21. Yu, P. B., Hong, C. C., Sachidanandan, C., Babitt, J. L., Deng, D. Y., Hoyng, S. A., Lin, H. Y., Bloch, K. D., and Peterson, R. T. (2008) *Nat. Chem. Biol.* **4,** 33–41
22. Yang, X., Li, C., Herrera, P. L., and Deng, C. X. (2002) *Genesis* **32,** 80–81
23. Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S., and Saito, I. (1995) *Nucleic Acids Res.* **23,** 3816–3821
24. Kodaira, K., Imada, M., Goto, M., Tomoyasu, A., Fukuda, T., Kamijo, R., Suda, T., Higashio, K., and Katagiri, T. (2006) *Biochem. Biophys. Res. Commun.* **345,** 1224–1231
25. Ohto, H., Kamada, S., Tago, K., Tominaga, S. I., Ozaki, H., Sato, S., and Kawakami, K. (1999) *Mol. Cell. Biol.* **19,** 6815–6824
26. Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K., and Ueno, N. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 10255–10259
27. Kao, K. R., and Elinson, R. P. (1988) *Dev. Biol.* **127,** 64–77
28. Hattori, H., Ishihara, M., Fukuda, T., Suda, T., and Katagiri, T. (2006) *Biochem. Biophys. Res. Commun.* **343,** 1118–1123
29. Zhao, B., Katagiri, T., Toyoda, H., Takada, T., Yanai, T., Fukuda, T., Chung, U. I., Koike, T., Takaoka, K., and Kamijo, R. (2006) *J. Biol. Chem.* **281,** 23246–23253
30. Hanai, J., Chen, L. F., Kanno, T., Ohtani-Fujita, N., Kim, W. Y., Guo, W. H., Imamura, T., Ishidou, Y., Fukuchi, M., Shi, M. J., Stavnezer, J., Kawabata, M., Miyazono, K., and Ito, Y. (1999) *J. Biol. Chem.* **274,** 31577–31582
31. Zhang, Y. W., Yasui, N., Ito, K., Huang, G., Fujii, M., Hanai, J., Nogami, H., Ochi, T., Miyazono, K., and Ito, Y. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 10549–10554
32. Ito, Y., and Miyazono, K. (2003) *Curr. Opin. Genet. Dev.* **13,** 43–47
33. Suzuki, H., Fukunishi, Y., Kagawa, I., Saito, R., Oda, H., Endo, T., Kondo, S., Bono, H., Okazaki, Y., and Hayashizaki, Y. (2001) *Genome Res.* **11,** 1758–1765
34. Pownall, M. E., Gustafsson, M. K., and Emerson, C. P., Jr. (2002) *Annu. Rev. Cell Dev. Biol.* **18,** 747–783
35. Akiyama, S., Katagiri, T., Namiki, M., Yamaji, N., Yamamoto, N., Miyama, K., Shibuya, H., Ueno, N., Wozney, J. M., and Suda, T. (1997) *Exp. Cell Res.* **235,** 362–369
36. Fujii, M., Takeda, K., Imamura, T., Aoki, H., Sampath, T. K., Enomoto, S., Kawabata, M., Kato, M., Ichijo, H., and Miyazono, K. (1999) *Mol. Biol. Cell* **10,** 3801–3813
37. McPherron, A. C., Lawler, A. M., and Lee, S. J. (1997) *Nature* **387,** 83–90
38. He, L., Vichev, K., Macharia, R., Huang, R., Christ, B., Patel, K., and Amthor, H. (2005) *Anat. Embryol.* **209,** 401–407
39. Massagué, J., and Wotton, D. (2000) *EMBO J.* **19,** 1745–1754
40. Le Cam, L., Lacroix, M., Ciemerych, M. A., Sardet, C., and Sicinski, P. (2004) *Mol. Cell. Biol.* **24,** 6467–6475
41. Rooney, R. J., Rothammer, K., and Fernandes, E. R. (1998) *Nucleic Acids Res.* **26,** 1681–1688
42. Lee, K. A., and Green, M. R. (1987) *EMBO J.* **6,** 1345–1353
43. Le Cam, L., Linares, L. K., Paul, C., Julien, E., Lacroix, M., Hatchi, E., Triboulet, R., Bossis, G., Shmueli, A., Rodriguez, M. S., Coux, O., and Sardet, C. (2006) *Cell* **127,** 775–788
44. Sato, M. M., Nashimoto, M., Katagiri, T., Yawaka, Y., and Tamura, M. (2009) *Biochem. Biophys. Res. Commun.* **383,** 125–129
45. Levine, A. J., Momand, J., and Finlay, C. A. (1991) *Nature* **351,** 453–456
46. Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996) *Science* **271,** 350–353
47. Clever, J. L., Sakai, Y., Wang, R. A., and Schneider, D. R. (2010) *Am. J. Physiol. Cell Physiol.* 10.1152/ajpcell.00388.2009
48. Tan, X., Weng, T., Zhang, J., Wang, J., Li, W., Wan, H., Lan, Y., Cheng, X., Hou, N., Liu, H., Ding, J., Lin, F., Yang, R., Gao, X., Chen, D., and Yang, X. (2007) *J. Cell Sci.* **120,** 2162–2170

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ORIGINAL ARTICLE

Curdlan Induces DC-Mediated Th17 Polarization via Jagged1 Activation in Human Dendritic Cells

Takehiro Higashi[1], Kumiko Hashimoto[1], Rie Takagi[1], Yosuke Mizuno[2], Yasushi Okazaki[2], Yoshiya Tanaka[3] and Sho Matsushita[1]

ABSTRACT

Background: Th17-inducing activity is carried by certain polysaccharides such as β-glucan derived from *Candia albicans*. Our previous studies have shown that Th1- and Th2-inducing activities can be qualitatively evaluated by the expression patterns of Notch ligand isoforms, using human monocyte-derived dendritic cells (Mo-DCs) and some leukemic cell lines such as THP-1. The association of Th17-inducing activities with Notch ligand expression patterns has been unclear.

Methods: Mo-DCs from healthy volunteers were co-cultured with HLA-DR-nonshared allogeneic $CD4^+$ naïve T cells to induce a mixed lymphocyte reaction, in the presence of adjuvants, such as curdlan. Culture supernatants were assayed for IFNγ, IL-5 and IL-17 by an enzyme-linked immunosorbent assay (ELISA). Notch ligand expression on Mo-DCs and THP-1 cells was evaluated by using RT-PCR.

Results: The present study shows that curdlan, one of the β-glucans, has the ability to induce DC-mediated Th17 differentiation. It is also interesting to note that Jagged1 mRNA in Mo-DCs and THP-1 cells is up-regulated by curdlan. Furthermore, polyclonal anti-Jagged1 antibody inhibited such DC-mediated Th17 differentiation.

Conclusions: This study suggests that curdlan induces human DC-mediated Th17 polarization via Jagged1 activation in DCs.

KEY WORDS

adjuvant, dendritic cells, Notch ligand, Th17, β-gulcan

INTRODUCTION

The different classes of specific immune responses are driven by the biased development of antigen-specific effector $CD4^+$ T-cell subsets such as Th1, Th2 and Th17 cells that activate different components of cellular and humoral immunity. The Th17 lineage characteristically produces high levels of IL-17, and it represents a significant revision of the Th1-Th2 paradigm.[1,2] Moreover, Th17 cells have been reported to play not only critical roles in the immune responses to extracellular microorganisms, such as fungi, but also a pathogenetic role in autoimmunity.[1,3-6] Dendritic cells (DCs) play a pivotal role in the differentiation of naïve CD4 T helper cells towards Th1 or Th2 cells. Environmental molecules, such as LPS, certain nucleic acids and fungus-derived glycoprotein molecules alter the DC function and thereby induce Th1 differentiation.[7] Because such DCs induce Th1 responses, they are designated DC1, and DC1-inducing molecules are called Th1 adjuvants. DC2 and Th2 adjuvants have also been reported. DCs matured in the presence of forskolin or prostaglandin E2 (PGE2) induce the differentiation of naïve CD4 T cells towards Th2.[8]

Notch signaling pathways are highly conserved in organisms ranging from invertebrates to mammals and they are involved in cell fate choice during development.[9] A correlation has also been observed between the Notch ligand mRNA levels in monocyte-

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derived DCs (Mo-DCs) and leukemic APCs with Th1/Th2 adjuvant activities.[10] Increased expression of Delta1 and Delta4 mRNA on DCs can predict Th2 and Th1 adjuvant activities, respectively. Indeed, in other studies using mouse models, Notch directly regulates Gata-3 expression during Th2 differentiation.[11,12]

The present study used a mixed lymphocyte reaction (MLR) between human monocyte-derived DCs and allogeneic naïve $CD4^+$ T cells to show that curdlan, one of the β-glucans, has the ability to induce DC-mediated Th17 differentiation. Jagged1 mRNA is up-regulated by curdlan. Moreover, polyclonal anti-Jagged1 antibody (Ab) inhibited such DC-mediated Th17 differentiation.

METHODS

PREPARATION OF HUMAN Mo-DCs, T LYMPHOCYTES AND PMA-DERIVED THP-1

This study using peripheral blood of healthy volunteers was approved by the Saitama Medical University Ethics Committee. Human $CD14^+$ cells were isolated from adult blood buffy coat specimens from healthy volunteer blood donors after the separation of peripheral blood mononuclear cells (PBMC) by Ficoll-Paque (GE Healthcare, Sweden) centrifugation and positive selection using CD14 MicroBeads (Miltenyi Biotec, Germany). These cells were further depleted using a $CD4^+$ T cell Isolation Kit (Miltenyi Biotec) and separated into $CD45RO^+$ memory T cells and $CD45RA^+$ naïve T cells using CD45RO MicroBeads (Miltenyi Biotec). A FACScan flow cytometer (Becton Dickinson, USA) showed the purity of these cells to be more than 99%. The $CD14^+$ cells were suspended in an RPMI 1640 medium containing 10% fetal calf serum (FCS), 1% L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 ng/ml of IL-4 (Primmune, Japan) and GM-CSF (Peprotech, USA) and then were cultured at 37℃ in a humidified atmosphere with 5% CO_2. After 5 days, the cells were harvested as immature Mo-DCs. To prepare THP-1-devided macrophage-like cells, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) as previously reported.[13]

DC-MEDIATED T CELL DIFFERENTIATION ASSAY

After washing, immature Mo-DCs were stimulated with curdlan purchased from Wako, Japan. In some experiments, immature Mo-DCs were pretreated with goat anti-human Jagged-1 Ab or goat IgG as a control for 1 hour before curdlan stimulation. Two days after the incubation with curdlan, cellular components were further co-cultured with HLA-DR-nonshared allogeneic $CD4^+$ naïve T cells to induce an MLR in RPMI 1640 medium supplemented with 10% human serum for 6-8 days. Thereafter, the T cells were restimulated with anti-CD3 and anti-CD28 Abs (BD Pharmingen, USA). Culture supernatants were harvested after 48 hours to be assayed for IFNγ, IL-5 and IL-17 by an enzyme-linked immunosorbent assay (ELISA) using IFNγ, IL-5 and IL-17 ELISA kits (R&D systems, USA).

STIMULATION OF APCs WITH ADJUVANTS

Mo-DCs were stimulated with curdlan, and PMA-treated THP-1 cells were stimulated with curdlan, laminarin (SIGMA, Japan) or zymosan (SIGMA) for 4 hours. Thereafter, the total RNA was extracted using TRIzol Reagent (Invitrogen Life Technologies, USA).

RT-PCR ANALYSIS OF NOTCH LIGAND GENE EXPRESSION

RNA was extracted using an RNeasy Mini kit (Qiagen, Germany), first-strand cDNA synthesis was performed using Omniscript reverse transcription for polymerase chain reaction (RT-PCR; Qiagen), and cDNA was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, USA). Primers of Notch ligand genes and β-actin[10] were synthesized as follows: Jagged1 sense 5'-AGTCACTGGCACGGTTG TAG-3', Jagged1 antisense 5'-TCGCTGTATCTGTCC ACCTG-3', Jagged2 sense 5'-GATTGGCGGCTATTA CTGTG-3', Jagged2 antisense 5'-AGGCAGTCGTCAA TGTTCTC-3', Delta1 sense 5'-AGACGGAGACCATG AACAAC-3' Delta1 antisense 5'-AGATGCTTCTCCCA CCCCTGA-3', Delta3 sense 5'-GTGAATGCCGATGC CTAGAG-3' Delta3 antisense 5'-GGTCCATCTGCAC ATGTCAC-3', Delta4 sense 5'-TGACCACTTCGGCCA CTATG-3', Delta4 antisense 5'-AGTTGGAGCCGGTG AAGTTG-3', β-actin sense 5'-CATCACCATTGGCAAT GAGC-3', β-actin sense 5'-CGATCCACACGGAGTAC TTG-3'.

Each PCR product was separated by electrophoresis on a 1.5% agarose gel, analyzed using the densitometer (BIO-RAD Laboratories, Japan) and the Jagged1/β-actin ratio was calculated. In some experiments, quantitative real time PCR was performed using Taqman gene expression systems (Applied Biosystems), a Jagged1 probe (ID: Hs01070036_m1) and a β-actin probe purchased from Applied Biosystems.

STATISTICAL ANALYSIS

Comparisons between sets of two groups were performed using Student's two-tailed *t*-test, while sets of more than two groups were compared by ANOVA.

RESULTS

DC-MEDIATED Th17 POLARIZATION BY CURDLAN

The cytokine profiles in the MLR induced by co-culture were determined in allogeneic $CD45RA^+$ naïve T cells and Mo-DCs stimulated with curdlan to test the induction of Th17 differentiation by human Mo-DCs stimulated with curdlan. As expected, 0.1-100 ng/ml curdlan led to IL-17 production. Stimulation at 1 ng/ml curdlan most markedly induced IL-17

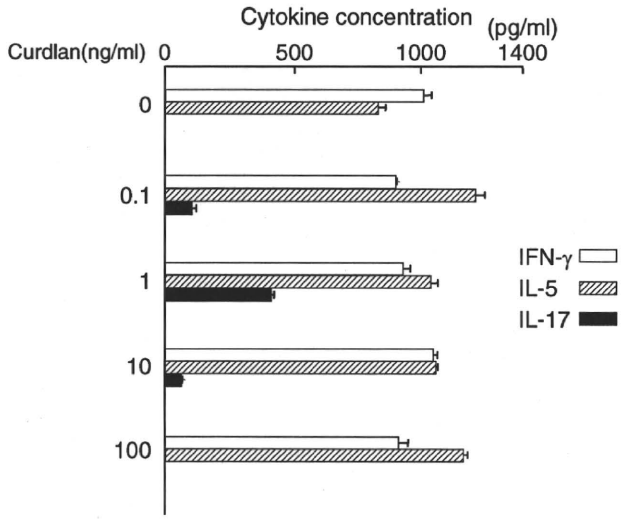


Fig. 1 DC-mediated Th17 polarization by curdlan. The $CD14^+$ cells were stimulated with IL-4 and GM-CSF for 5 days and the cells were harvested as immature Mo-DCs. After washing, immature Mo-DCs were incubated with 0-100 ng/ml of curdlan. Two days after the incubation with curdlan, cellular components were further co-cultured for 7 days with HLA-DR-nonshared allogeneic $CD4^+$ naïve T cells to induce an MLR. Thereafter, the T cells were restimulated with anti-CD3 and anti-CD28 Abs. The culture supernatants were harvested after 48 hours to be assayed for IFNγ, IL-5 and IL-17 by an ELISA.

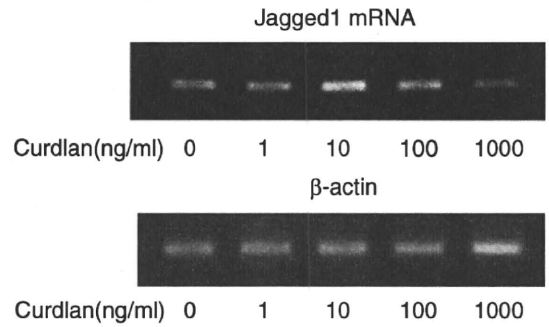


Fig. 2 The expression patterns of Notch ligand mRNA in curdlan-stimulated Mo-DCs. Mo-DCs were stimulated with curdlan for 4 hours. After stimulation, the expressions of Jagged1 mRNA and β-actin were evaluated by RT-PCR. The primers were described in "Methods". PCR products were separated by electrophoresis on a 1.5% agarose gel.

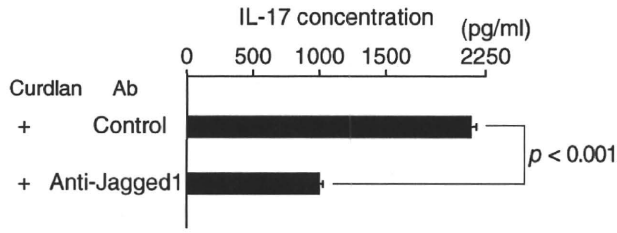


Fig. 3 Inhibition of DC-mediated Th17 polarization by polyclonal anti-Jagged1 Ab. Immature Mo-DCs were treated with 1 μg/ml of polyclonal anti-Jagged1 Ab or a control for 1 hour before curdlan stimulation (1 ng/ml). Two days after the incubation with curdlan, cellular components were further co-cultured with HLA-DR-nonshared allogeneic $CD4^+$ naïve T cells to induce an MLR. Thereafter, the T cells were restimulated with anti-CD3 and anti-CD28 Abs. The culture supernatants were harvested after 48 hours to be assayed for IL-17 by an ELISA.

production (Fig. 1). In contrast, curdlan had no effect on IFN-γ or IL-5 production.

INCREASED JAGGED1 mRNA LEVEL IN Mo-DCs WAS INDUCED BY CURDLAN-STIMULATION IN THE Mo-DCs

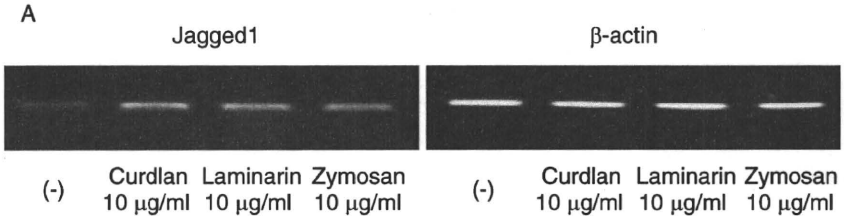
Next, the expression patterns of Notch ligand mRNA were examined in curdlan-stimulated Mo-DCs, using RT-PCR. Figure 2 shows that 10-100 ng/ml curdlan induced increased Jagged1 mRNA in Mo-DCs. None of the other Notch ligands, i.e., Delta1, Delta3, Delta4 or Jagged2 mRNA were below the limit of detection in our PCR systems (data not shown). Experiments were repeated six times and the stimulation with 10 ng/ml, but not 100 ng/ml curdlan reproducibly up-regulated Jagged1 mRNA in Mo-DCs. The Jagged1/β-actin ratio ranged from 1.3 to 1.42 ($p < 0.05$). The expression was also examined at the protein level by Western blotting and/or flow cytometry. However, antibodies capable of specifically detecting human Jagged1 without cross reactivity to other ligands are not commercially available at present.

POLYCLONAL ANTI-JAGGED1 AB INHIBITED Th17 DIFFERENTIATION

The inhibitory effect of polyclonal anti-Jagged1 antibody was examined to confirm that Jagged1 has a functional role in curdlan-mediated Th17 differentiation. Mo-DCs were incubated with anti-Jagged1 Ab for 1 hour before the addition of curdlan to the culture, followed by MLR as described in Figure 1. As shown in Figure 3, IL-17 production was significantly inhibited by anti-Jagged1 Ab in comparison to a control ($p < 0.001$).

Experiments were reproducibly repeated three times. We also observed a slight increase of IFNγ and a slight decrease of IL-5, by using anti-Jagged1 Ab (not shown), which will be discussed later in the discussion section. MLR is caused by co-culturing Mo-DCs with polyclonal $CD4^+$ naïve T cells. The clonal frequency of alloreactive T cells is only 10^{-4} to 10^{-3}, which resulted in very high background signals for transcriptional factors caused by non-reactive T cells. It was thereby difficult to show whether stimulating

A



B

Quantitative real-time PCR

	(-)	Curdlan 10 mg/ml	Laminarin 10 mg/ml	Zymosan 10 mg/ml
Jagged1	1.14 ± 0.03	1.37 ± 0.06	1.33 ± 0.3	1.31 ± 0.1

Fig. 4 The expression patterns of Notch ligand mRNA in the curdlan-stimulated human leukemic APC line, THP-1. THP-1 cells were incubated with PMA for 2 days to induce macrophage-like cells. PMA-derived THP-1 cells were stimulated with curdlan, laminarin or zymosan for 4 hours. **A.** After stimulation, expression of Jagged1 mRNA and β-actin were evaluated by RT-PCR. The primers were described in "Methods". PCR products were separated by electrophoresis on a 1.5% agarose gel. **B.** Expression of Jagged1 mRNA and β-actin were evaluated by quantitative real-time PCR. The probes were described in "Methods".

Notch directly regulates GATA, RORγt or Foxp3 by RT-PCR in this MLR system.

INCREASED JAGGED1 mRNA LEVEL WAS INDUCED BY β-GLUCAN-STIMULATION IN PMA-DERIVED THP-1

Previous studies showed that Delta1/Delta4 gene expression patterns in human leukemic APCs have a potential to evaluate Th1/Th2 adjuvant activities.[10] We therefore observed all Notch ligand isoforms in APC lines stimulated with β-glucans. As shown in Figure 4A, β-glucans, such as curdlan, zymosan and laminarin induced increased Jagged1 mRNA in PMA-derived THP-1 cells. Experiments were repeated six times and the stimulation with 100 μg/ml β-glucans reproducibly up-regulated Jagged1 mRNA in Mo-DCs. The Jagged1/β-actin ratio ranged from 1.4 to 1.5 ($p < 0.01$). Other Notch ligands exhibited no change in their expression (data not shown). Next, we observed Jagged1 mRNA in PMA-derived THP-1 cells using quantitative real-time PCR studies. As shown in Figure 4B, the stimulation with 10 μg/ml curdlan, zymosan or laminalin up-regulated Jagged1 mRNA in PMA-derived THP-1 cells ($p < 0.01$).

DISCUSSION

The present study used a mixed lymphocyte reaction (MLR) between human monocyte-derived DCs and allogeneic naïve $CD4^+$ T cells to show that curdlan, one of β-glucans, has the ability to induce DC-mediated Th17 differentiation. Jagged1 mRNA is up-regulated by curdlan. Moreover, polyclonal anti-Jagged1 antibody inhibited this DC-mediated Th17 differentiation. These findings suggest that curdlan induces human DC-mediated Th17 polarization via Jagged1 activation in DCs. Th17 responses could be observed by using human PBMC stimulated with various exogenous antigens, such as *Candida albicans*[6,14] and purified protein derivative. This observation raises the possibility that particular exogenous crude antigens stimulate the differentiation of naïve CD4 T cells. Specific adjuvant activities have been extensively described, for Th1 and Th2 responses, which are often associated with maturation and differentiation of DCs towards DC1/DC2.[15] Zymosan, one of the β-glucans, binds to dectin-1, and leads to the activation of Syk followed by the production of IL-23 from APC and IL-17 from T cells.[16] In the present study, not only polyclonal anti-Jagged1 Ab (Fig. 3) but also polyclonal anti-dectin-1 Ab inhibited Mo-DCs-mediated Th17 polarization by curdlan (data not shown). These findings collectively suggest that a consecutive signaling pathway, including β-glucan, dectin-1 and Jagged1, plays an important role in Mo-DCs maturation, thus resulting in Th17 differentiation via the Jagged1-Notch pathway. β-glucans, such as curdlan, zymosan and laminarin, can also trigger severe chronic arthritis in SKG mice, which is one of Th17-mediated disease models.[17,18] Laminarin and zymosan have been tested as well, and we successfully proved that they also carry the Th17-inducing and Jagged1-inducing activities (data not shown). We also observed a slight increase of IFNγ accompanied by a slight decrease of IL-5, by using anti-Jagged1 Ab. This might be caused by signaling mediators, such as cAMP, which is shared by Th2 and Th17 differentia-

tion pathways as shown in our previous studies.[19-21]

A correlation has been reported between the Notch ligand mRNA levels of Mo-DCs and leukemic APCs with Th1/Th2 adjuvant activities.[10] An increased expression of Delta1 and Delta4 mRNA in DCs can predict the Th2 and Th1 adjuvant activities, respectively. In this study, curdlan increased expression of Jagged1 mRNA level but not the other Notch ligands. The same results were obtained from analysis using human leukemic APCs, THP-1, as reported in previous Th1/Th2 adjuvant studies.[13] Although an increased expression of Jagged1 mRNA was most efficiently induced by 10 ng/ml curdlan in Mo-DCs, 10 μg/ml of curdlan was needed in PMA-stimulated THP-1 cells. This discrepancy may be attributable to the fact that PMA-stimulated THP-1 cells are not physiological DCs. Our results suggested that increased expression of Jagged1 mRNA in Mo-DCs or leukemic APCs can predict Th17 adjuvant activity. Furthermore, polyclonal anti-Jagged1 Ab inhibited Mo-DCs-mediated Th17 polarization by curdlan. Indeed, in other studies using mouse models, Notch directly regulates the Gata-3 expression during Th2 differentiation.[11,12] These data collectively suggest that Jagged1 is not only a marker for DC17 but also a functional molecule for the induction of Th17 differentiation.

The detection of Th17 adjuvant activities has long been dependent on an assay for the cytokine profiles induced on T cells by co-culturing with APCs stimulated with adjuvants. This assay requires a large amount of labor and more than 2 weeks due to the preparation of cells from healthy donors and a large number of culture processes. In addition, the heterogeneities of cell sources and donor-to-donor variances may also lead to difficulties in obtaining stable and reproducible results. The present study demonstrated, for the first time, that increased Jagged1 mRNA in PMA-derived THP-1 cells have the potential to scrutinize and evaluate Th17 adjuvant candidates out of a large number of environmental substances and natural products, without possible instability arising from cell sources and polymorphisms. It is also interesting to note that this assay system can be used for the evaluation of live microorganisms, because 4-hour incubation with live probiotic bacteria or *Candida albicans* did not decrease the viability of the THP-1 cells, while successfully inducing Delta1/Delta4 and Jagged1, respectively (data not shown).[10] We are currently scrutinizing bacteria and fungi that carry Th17-inducing activity, which might be associated with infection-related autoimmunity.

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REFERENCES

1. Park H, Li Z, Yang XO *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005;**6**:1133-41.
2. Harrington LE, Hatton RD, Mangan PR *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;**6**:1123-32.
3. Nakae S, Nambu A, Sudo K, Iwakura Y. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. *J Immunol* 2003;**171**:6173-7.
4. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006;**24**:179-89.
5. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007;**25**:821-52.
6. Acosta-Rodriguez EV, Rivino L, Geginat J *et al.* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007; **8**:639-46.
7. Kaisho T, Akira S. Toll-like receptors as adjuvant receptors. *Biochim Biophys Acta* 2002;**1589**:1-13.
8. Kalinski P, Schuitemaker JH, Hilkens CM, Kapsenberg ML. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J Immunol* 1998;**161**:2804-9.
9. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* 1999;**284**:770-6.
10. Wakui M, Nakano K, Matsushita S. Notch ligand mRNA levels of human APCs predict Th1/Th2-promoting activities. *Biochem Biophys Res Commun* 2007;**358**:596-601.
11. Amsen D, Antov A, Jankovic D *et al.* Direct regulation of gata3 expression determines the T helper differentiation potential of notch. *Immunity* 2007;**27**:89-99.
12. Fang TC, Yashiro-Ohtani Y, Del Bianco C, Knoblock DM, Blacklow SC, Pear WS. Notch directly regulates Gata3 expression during T helper 2 cell differentiation. *Immunity* 2007;**27**:100-10.
13. Higashi T, Wakui M, Nakano K *et al.* Evaluation of adjuvant activities using human antigen presenting cells in vitro. *Allergol Int* 2008;**57**:219-22.
14. Takagi R, Higashi T, Hashimoto K *et al.* B cell chemoattractant CXCL13 is preferentially expressed by human Th17 cell clones. *J Immunol* 2008;**181**:186-9.
15. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 1999; **20**:561-7.
16. LeibundGut-Landmann S, Gross O, Robinson MJ *et al.* Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol* 2007;**8**:630-8.
17. Yoshitomi H, Sakaguchi N, Kobayashi K *et al.* A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J Exp Med* 2005;**201**:949-60.
18. Hirota K, Hashimoto M, Yoshitomi H *et al.* T cell self-reactivity forms a cytokine milieu for spontaneous devel-

opment of IL-17+ Th cells that cause autoimmune arthritis. *J Exp Med* 2007;**204**:41-7.
19. Nakano K, Higashi T, Hashimoto K, Takagi R, Tanaka Y, Matsushita S. Antagonizing dopamine D1-like receptor inhibits Th17 cell differentiation: preventive and therapeutic effects on experimental autoimmune encephalomyelitis. *Biochem Biophys Res Commun* 2008;**373**:286-91.
20. Nakano K, Higashi T, Takagi R, Hashimoto K, Tanaka Y, Matsushita S. Dopamine released by dendritic cells polarizes Th2 differentiation. *Int Immunol* 2009;**21**:645-54.
21. Hashimoto K, Inoue T, Higashi T *et al.* Dopamine D1-like receptor antagonist, SCH23390, exhibits a preventive effect on diabetes mellitus that occurs naturally in NOD mice. *Biochem Biophys Res Commun* 2009;**383**:460-3.

Surgical treatment of gastric cancer: 15-year follow-up results of the randomised nationwide Dutch D1D2 trial

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Background Historical data and recent studies show that standardised extended (D2) lymphadenectomy leads to better results than standardised limited (D1) lymphadenectomy. Based on these findings, the Dutch D1D2 trial, a nationwide prospectively randomised clinical trial, was undertaken to compare D2 with D1 lymphadenectomy in patients with resectable primary adenocarcinoma of the stomach. The aim of the study was to assess the effect of D2 compared with D1 surgery on disease recurrence and survival in patients treated with curative intent.

Methods Between August, 1989, and July, 1993, patients were entered and randomised at 80 participating hospitals by means of a telephone call to the central data centre of the trial. The sequence of randomisation was in blocks of six with stratification for the participating centre. Eligibility criteria were a histologically proven adenocarcinoma of the stomach without evidence of distance metastasis, age younger than 85 years, and adequate physical condition for D1 or D2 lymphadenectomy. Patients were excluded if they had previous or coexisting cancer or had undergone gastrectomy for benign tumours. Strict quality control measures for pathological assessment were implemented and monitored. Analyses were by intention to treat. This study is registered with the NCI trial register, as DUT-KWF-CKVO-8905, EU-90003.

Findings A total of 1078 patients were entered in the study, of whom 996 were eligible. 711 patients underwent the randomly assigned treatment with curative intent (380 in the D1 group and 331 in the D2 group) and 285 had palliative treatment. Data were collected prospectively and all patients were followed up for a median time of 15·2 years (range 6·9–17·9 years). Analyses were done for the 711 patients treated with curative intent and were according to the allocated treatment group. Of the 711 patients, 174 (25%) were alive, all but one without recurrence. Overall 15-year survival was 21% (82 patients) for the D1 group and 29% (92 patients) for the D2 group (p=0·34). Gastric-cancer-related death rate was significantly higher in the D1 group (48%, 182 patients) compared with the D2 group (37%, 123 patients), whereas death due to other diseases was similar in both groups. Local recurrence was 22% (82 patients) in the D1 group versus 12% (40 patients) in D2, and regional recurrence was 19% (73 patients) in D1 versus 13% (43 patients) in D2. Patients who had the D2 procedure had a significantly higher operative mortality rate than those who had D1 (n=32 [10%] *vs* n=15 [4%]; 95% CI for the difference 2–9; p=0·004), higher complication rate (n=142 [43%] *vs* n=94 [25%]; 11–25; p<0·0001), and higher reoperation rate (n=59 [18%] *vs* n=30 [8%]; 5–15; p=0·00016).

Interpretation After a median follow-up of 15 years, D2 lymphadenectomy is associated with lower locoregional recurrence and gastric-cancer-related death rates than D1 surgery. The D2 procedure was also associated with significantly higher postoperative mortality, morbidity, and reoperation rates. Because a safer, spleen-preserving D2 resection technique is currently available in high-volume centres, D2 lymphadenectomy is the recommended surgical approach for patients with resectable (curable) gastric cancer.

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Introduction

After several decades of debate on what the optimum surgical treatment of gastric cancer should be, it is now possible to treat patients using evidence-based principles established by well designed and conducted studies. Adequate surgery, the only treatment known to offer cure, is still the cornerstone of gastric-cancer treatment; however, local regional control remains an issue. In western Europe and the USA, optimum local control and survival seemed to be reached with surgery as a single-modality treatment, based mainly on two large European trials, the Dutch Gastric Cancer Trial (DGCT)[1] and the UK Medical Research Council (MRC) randomised trial.[2] In both trials, standardised extended (D2) lymphadenectomy did not improve survival, and was associated with significantly higher morbidity and mortality compared with standardised limited (D1) lymphadenectomy. The unfavourable outcomes were mostly associated with pancreatico-splenectomy, which was an integral part of the D2 resection in both trials. In 2004, results from a study by Degiuli and colleagues[3] suggested a survival benefit after pancreas-preserving D2 resections, and in 2006, a Taiwanese single-institution trial[4] found that extended lymph-node dissection (D2) led to better results (no postoperative mortality) than D1 lymphadenectomy. More extended resection (D2 plus para-aortic nodal dissection) was not found to be better than D2 resections in Japanese patients.[5]

As a result of the INT0116 trial,[6] a combination of surgery and postoperative chemoradiotherapy became the standard treatment for curable gastric cancer in the USA. In this trial, fluorouracil plus leucovorin given concomitantly with 45 Gy radiation after potentially curative surgery improved 3-year survival from 41% to 50%, compared with surgery alone. In Europe, following the results of the UK MAGIC trial,[7] perioperative chemotherapy with the ECF (epirubicin, cisplatin, and fluorouracil) became the new treatment standard for gastric cancer. The MAGIC trial found that perioperative systemic chemotherapy improved 5-year survival from 23% to 36%, compared with surgery alone. Randomised controlled trials in Japanese patients have shown significant improvement in overall survival with postoperative adjuvant chemotherapy with S-1 (an orally active combination of tegafur, gimeracil, and oteracil) after D2 dissection.[8] Therefore, S-1 after D2 surgery is becoming the standard treatment for patients with gastric cancer in Japan.

Early results from the DGCT showed significantly higher postoperative morbidity and mortality in the D2 group compared with the D1 group.[9] With the 5-year follow-up results showing no significant survival benefit in the D2 group, a conclusion was drawn that D2 resection could not be advised in patients with curable gastric cancer.[10] However, 11-year follow-up data showed better survival results in exploratory analyses in patients with stage II and IIIa disease who had D2 compared with D1 resections.[1] The current report is the 15-year follow-up data of the DGCT.

Methods

Patients

The DGCT was approved by the medical ethics committees of the Leiden University Medical Center and all participating hospitals. Written or oral informed consent was obtained according to the principles of the institution. 80 hospitals participated in the trial. Eligible patients had histologically proven adenocarcinoma of the stomach without evidence of distance metastasis, were younger than 85 years, and were in adequate physical condition for D1 or D2 lymphadenectomy. Patients were excluded if they had previous or coexisting cancer or had undergone gastrectomy for benign tumours. Randomisation was done before surgery to allow scheduling for the presence of specially trained supervising surgeons. If a supervising surgeon could not attend a planned surgery, the patient was considered ineligible. No patients received adjuvant chemotherapy, because at the time of the trial chemotherapy was not standard therapy.

All patients were assessed every 3 months during the first year and every 6 months thereafter. In accordance with common practice at that time, a clinical diagnosis was considered sufficient evidence of recurrence; however, radiological or endoscopic confirmation was sought for most patients. Evidence of recurrent disease was accepted only if one of the following criteria were present: cytological puncture under CT scan or ultrasonography guidance; local recurrence found by endoscopy or with relaparotomy; peritoneal dissemination on ultrasonography or CT scan; liver metastasis on ultrasonography or CT scan; distant metastasis, including supraclavicular lymph-node involvement or metastases in the Douglas pouch; lung metastasis on chest radiography; or bone metastasis on radiography or bone scan. After recurrence, no data are available on additional treatments, and only the date of death was registered. Post-mortem examination was desired to confirm death due to disease, although not all patients had a post-mortem examination.

Randomisation and masking

After establishing the diagnosis and curative resectability, participating centres registered patients by means of a telephone call to the central data centre of the trial at the Leiden University Medical Center, Germany. The following information was requested: the institution's name, patient's name and date of birth, surgeon's name, and a check of inclusion and exclusion criteria. The sequence of randomisation was in blocks of six with stratification according to the participating centre. The surgical procedure (D1 or D2) was then assigned and a date for the surgery was agreed on. After registration, reference to the case was by a reference number, thus ensuring the privacy of the patient. Because of the nature of the treatment, concealment from the surgeons was not possible; the surgeons assessing outcomes or analysing data were not masked to group assignment.

Procedures

A D1 dissection entailed removal of the involved part of the stomach or the entire stomach (distal or total resection), including the perigastric lymph nodes (N1 level, station numbers 1–6) and the greater and lesser omenta. For a D2 dissection, both N1 and N2 lymph nodes (station numbers 7–11) were removed, along with omental bursa and the front leaf of the transverse mesocolon (figure 1). At the time of the trial, resection of the spleen and pancreatic tail were regarded as necessary for adequate removal of D2 lymph-node stations 10 and 11 in proximal tumours, and in D1 in the case of tumour invasion. Station numbers 12–14 were grouped as N3 and station numbers 15 and 16 as N4, but were outside the scope of our trial because involvement of N3 and N4 were considered distant metastases.

Assessment of curability was done by the supervising surgeon at laparotomy. Patients were regarded as able to undergo resection with curative intent and underwent the randomly assigned treatment (D1 or D2) if, at laparotomy, they had a macroscopically completely

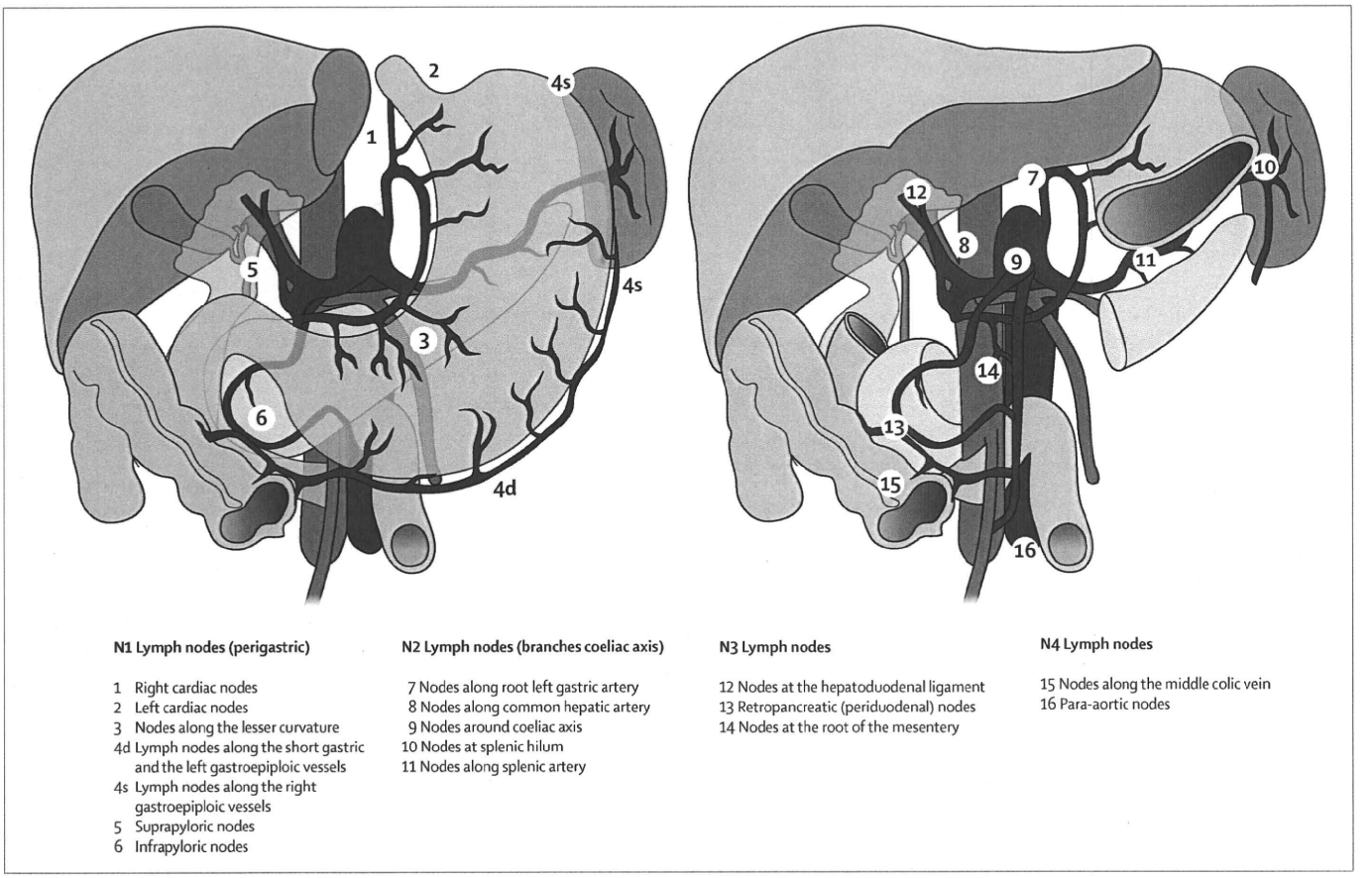

Figure 1: **Location and grouping of the lymph nodes**
D1 resection: removal of the N1 lymph nodes. D2 resection: removal of the N1 and N2 lymph nodes. Courtesy of Cornelis van de Velde.

removable tumour without peritoneal spread, liver metastases, or distant lymph-node metastases. For the latter criterion, a frozen-section examination of one or two para-aortic lymph nodes was required. Patients who met these criteria constituted the group treated with curative intent. To detect free abdominal tumour cells, abdominal fluid obtained by irrigation of the abdominal cavity immediately after laparotomy was recommended. The results were not used for immediate assessment of curability. The type of gastrectomy performed (distal or total) was independent of randomisation. Distal gastrectomy was allowed if there was a tumour-free margin of 5 cm beyond the proximal resection line. All other patients had total gastrectomy. Reconstruction of the alimentary tract was done mainly by the local surgeon, who used their preferred method. Histological examination of the resected specimens was done by the local pathologist, and the results were reviewed by a panel of supervising pathologists. After the final pathological examination, resections were considered R0 (radical resection, as opposed to R1, microscopic remnant tumour, and R2, macroscopic remnant tumour) when the cytology of the abdominal washing fluid was tumour negative, the resection lines were microscopically tumour negative, no distant lymph-node stations (beyond N2) were involved, and there were no distant metastases. Patients who did not meet these criteria constituted the group treated non-curatively. These patients underwent a palliative surgical procedure or exploratory laparotomy at the discretion of the surgeon and irrespective of the assigned treatment. None of the patients treated curatively underwent adjuvant radiotherapy or chemotherapy.

The primary endpoint of the trial was overall survival, calculated from the day of randomisation until the day of death (event) or the day of last follow-up (censored) and recurrence, defined as the time from randomisation to disease recurrence; the data of a patient were censored when at last follow-up the patient was alive with no evidence of disease, or had died of diseases other than gastric cancer without evidence of a recurrence. Disease-free survival was defined as the time from randomisation to recurrence or death due to any cause. All analyses were based on patients treated with curative intent and were according to the group to which patients were randomised.

	D1 group	D2 group
Randomised	539	539
Ineligible	26	56
No supervising surgeon available	5	30
Metastases or second tumour	9	11
No adenocarcinoma	8	10
Physical condition	4	6
Eligible	513	483
Non-curative*	133	152
Remnant tumour	90	102
Peritoneal metastases	59	83
Distant lymph-node metastases	36	37
Liver metastases	27	22
Curative	380	331

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. *Patients may be included in more than one category.

Table 1: **Numbers of patients in the Dutch Gastric Cancer Trial**

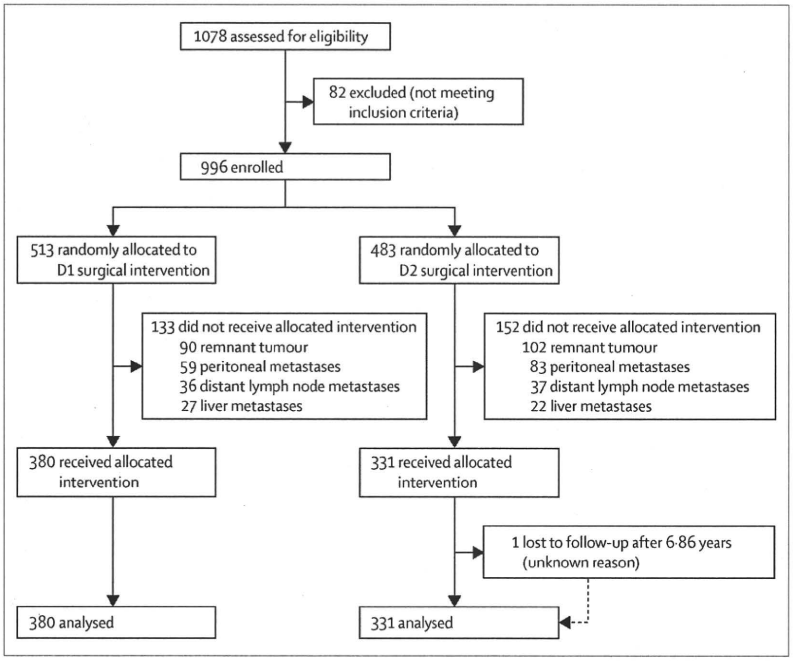

Figure 2: **Trial profile**

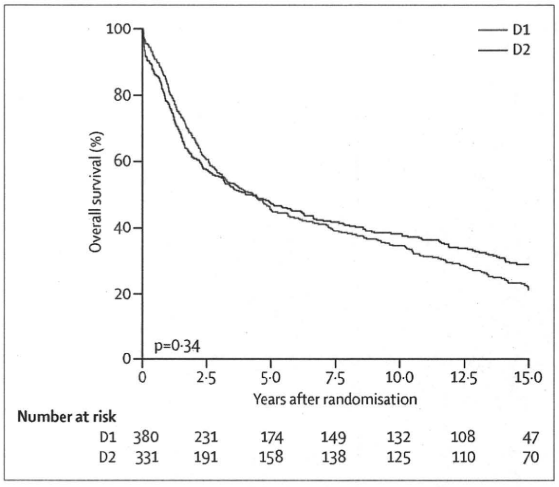

Figure 3: **Overall survival in patients treated with curative intent (n=711)**
D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy.

Strict quality control measures for surgery and pathology were implemented and monitored. Participating surgeons received a videotape and an instruction booklet about the technique and were instructed in the operating theatre by an expert gastric-cancer surgeon. The expert was present during the first 4 months of intake, which served as an instruction period, and regularly thereafter. All surgeries involving D2 dissection were attended by one of 11 surgeons who had been trained in D2 dissection. The study coordinator attended nearly all D1 procedures. The supervising surgeons monitored the technique and the extent of lymph-node dissection. After the surgery, the perigastric tissue was divided into lymph-node stations and fresh specimens were sent to the pathologist. All locations dissected not en-bloc were prepared and labelled by the surgeon. Regular meetings about the technique were held with the supervising surgeons, the study coordinator, and the instructing surgeon. Quality control was done by relating the number and location of the lymph nodes detected at pathological examination to the guidelines of the study protocol.[1,9,10]

Statistical analysis

The study size was based on an expected 5-year survival of 20% for patients undergoing D1 dissection with curative intent and 32% for those undergoing D2 dissection with curative intent. Using a two-sided significance level of 5%, a power of 90%, and an expected curability rate of 40%, 1062 patients had to be randomised.

The SPSS program (SPSS Inc, Chicago, IL, USA) and R version 2.9.1 were used for statistical analysis. A two-sided p value of 0·05 was considered statistically significant. Logistic regression was used to assess the influence of prognostic factors on postoperative mortality. The χ^2 test was applied to assess differences in proportions, and the log-rank test was used to assess the difference in survival and recurrence rates between groups, although the assumption of proportional hazards was not always satisfied.[11] The Kaplan-Meier method was used to estimate survival curves for overall and disease-free survival. For the analysis of death due to gastric cancer and due to other causes, a competing-risks analysis was done. Cumulative incidences were calculated and Gray's test was done to test between treatment groups.[12] Hazard ratios reported for these competing-risks analyses are based on the Fine and Gray model.[13]

	D1 group (n=380)	D2 group (n=331)
R0 resection	339	297
Cytology tumour-positive	15	10
Resection-line involvement		
Proximal	10	13
Distal	15	9
Distant metastases	8	3
Distant lymph-node involvement		
Station # 12	1	3
Station # 13	2	4
Station # 14	2	0
Station # 16	8	0
Total number of patients excluded	41	34

Patients may be included in more than one category. D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. R0=radical resection. R1=microscopic remnant tumour.

***Table 2:* Reasons for R1 rather than R0 resections**

	D1 group		D2 group		HR (95% CI)	Log-rank p value	Interaction p value
	N	15-year overall survival (95% CI)	N	15-year survival overall (95% CI)			
Total	380	21% (17–26)	331	29% (24–34)	0·92 (0·78–1·09)	0·34	..
Sex							
Male	215	21% (15–27)	186	24% (18–30)	1·08 (0·86–1·35)	0·50	0·04
Female	165	21% (15–28)	145	35% (27–43)	0·75 (0·58–0·98)	0·03	
Age, years							
≤70	252	30% (25–36)	229	36% (30–42)	0·93 (0·75–1·16)	0·54	0·89
>70	128	3% (0–6)	102	13% (6–20)	0·88 (0·67–1·16)	0·36	
Pathological stage*							
T1	98	39% (29–49)	85	53% (42–63)	0·79 (0·53–1·18)	0·25	0·79
T2	181	21% (14–27)	152	25% (18–32)	0·95 (0·75–1·22)	0·70	
T3	94	5% (1–10)	82	15% (7–22)	0·94 (0·68–1·28)	0·68	
Lymph nodes							
Negative	171	35% (27–42)	144	39% (31–47)	0·98 (0·74–1·30)	0·88	0·33
Positive	209	10% (6–14)	187	22% (16–27)	0·87 (0·70–1·08)	0·20	
N stage							
N0	171	35% (27–42)	144	39% (31–47)	0·98 (0·74–1·30)	0·88	0·15
N1	138	15% (9–21)	113	28% (19–36)	0·87 (0·66–1·15)	0·33	
N2	50	0% (0)	47	19% (8–30)	0·68 (0·44–1·04)	0·07	
N3	21	0% (0)	27	0% (0)	0·73 (0·40–1·25)	0·28	
TNM stage (UICC, 1997)†							
IA	75	41% (29–52)	69	53% (42–65)	0·79 (0·50–1·25)	0·32	0·14
IB	97	36% (26–45)	72	27% (17–38)	1·29 (0·89–1·85)	0·18	
II	93	15% (7–22)	77	33% (23–44)	0·68 (0·48–0·97)	0·03	
IIIA	60	3% (0–8)	54	19% (8–29)	0·84 (0·57–1·25)	0·39	
IIIB	24	0% (0)	20	10% (0–23)	0·81 (0·44–1·51)	0·51	
IV	28	0% (0)	36	3% (0–8)	0·71 (0·42–1·18)	0·18	
Gastrectomy							
Total	115	15 (9–22)	126	19 (12–26)	1·00 (0·76–1·32)	0·99	0·35
Partial	265	24 (18–29)	205	35 (29–42)	0·82 (0·66–1·02)	0·08	

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. HR=hazard ratio. TNM=tumour, node, metastasis. UICC=International Union Against Cancer. *Does not include two patients with missing data, two patients with T0 stage tumours, and three patients with T4 stage tumours in the D1 group, and three patients with T0 stage tumours and nine patients with T4 stage tumours in the D2 group. †Does not include one patient with missing data and two patients with TNM0 in the D1 group, and three patients with TNM0 in the D2 group.

***Table 3:* Univariate analysis of overall survival at 15 years (median) follow-up**

Risk of recurrence is also reported as cumulative incidence, accounting for death due to other causes as a competing risk.

For the subgroup analysis, no adjustment for multiple testing was applied. Interpretation of the results of subset analyses have to be judged carefully and any significant results should be viewed as hypotheses that require validation in subsequent studies. A p value of 0·05 might not be strict enough for these subgroups. Cox proportional-hazards model was used to test for interaction between prognostic factors and lymph-node dissection.

This study is registered with the NCI trial register, identifier DUT-KWF-CKVO-8905, EU-90003.

Role of the funding source

The funding source had no role in the study design, collection, analysis, or interpretation of data, or writing of the report. The corresponding author had full access to all data and the final responsibility to submit for publication.

Results

Between August, 1989, and July, 1993, 1078 patients with gastric adenocarcinoma were entered and randomised (539 to each group) in the DGCT. 996 patients met the eligibility criteria and were randomly assigned to have a D1 or D2 lymph-node dissection. Because of peritoneal, hepatic, or distant lymph-node metastasis or locally irresectable disease, 285 patients (29%) underwent palliative surgery without a formal lymph-node dissection, according to the discretion of the surgeon. Of these 285 patients, 156 (55%) had a palliative resection, 51 (18%) had a bypass, and 78 (27%) had exploration only. Characteristics of the patients judged ineligible and non-curative are summarised in table 1 and patient flowchart in figure 2. The remaining 711 patients underwent a D1 or D2 resection with curative intent, according to random assignment. Data were collected prospectively and all patients were followed up: median follow-up for all eligible patients was 15·2 years (range 6·9–17·9). The lower limit is 6·9 years because one patient was lost to follow-up at that point. The trial is now closed. This analysis focuses on the 711 patients (71%) who had a curative resection with D1 (n=380) or D2 (n=331) lymphadenectomy. The characteristics of the 711 curative patients are well balanced between the two treatment groups, except for pancreatico-splenectomy, which was expected because of the protocol and type of gastrectomy (webappendix pp 1[9,10]).

See Online for webappendix

At last follow-up, 217 patients (31%) died without recurrence (110 in the D1 group and 107 in the D2 group)

	D1 group (n=380)	D2 group (n=331)	p value
Alive	82 (22%)	92 (28%)	0·34*
Deaths from gastric cancer	182 (48%)	123 (37%)	0·01†
Deaths from other causes	116 (31%)	116 (35%)	0·12†
Other diseases	94 (25%)	77 (23%)	..
Toxicity treatment	15 (4%)	32 (10%)	..
Unknown	7 (2%)	7 (2%)	..

Data are number of patients (%) or p value. D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. *Log-rank p value. †Gray's test p value.

Table 4: **Causes of death**

and 320 patients (45%) died with recurrence (188 in D1 and 131 in D2). 173 patients (24%) were alive without recurrence (82 in D1 and 91 in D2), and one patient was alive with recurrence in the D2 group. 15-year overall survival for patients who had curative resections was 21% (85 of 380, 95% CI 17–26) for D1 and 29% (98 of 331, 24–34) for D2 (log-rank p=0·34; figure 3).

D2 patients had significantly higher operative mortality than D1 patients (32 of 331 [10%] *vs* 15 of 380 [4%], 95% CI for the difference 2–9%, p=0·004), higher complication rate (142 of 331 [43%] *vs* 94 of 380 [25%], 11–25%, p<0·0001, and higher reoperation rate (59 of 331 [18%] *vs* 30 of 380 [8%] 5–15%, p<0·00016; previously published[9,10]). Significant risk factors for postoperative mortality in logistic regression analysis were: age >70 versus ≤70 years (overall risk [OR] 3·55, 95% CI 1·91–6·61, p<0·0001), D2 versus D1 lymphadenectomy (OR 2·64, 1·38–5·04, p=0·003), male versus female (OR 2·50, 1·25–4·99, p=0·01) and total versus partial gastrectomy (OR 2·04, 1·01–3·79, p=0·02; data not shown). Other factors included in the analysis but that had no significant association with postoperative mortality were T stage, lymph-node involvement (N-negative *vs* N-positive and N0–N3), and TNM stage. Overall survival for patients who had an R0 resection was 25% (85 of 324, 95% CI 20–30) for D1 and 35% (97 of 268, 30–41) for D2, when postoperative deaths (15 of 380 [4%] in D1 and 32 of 331 [10%] in D2) are excluded (log-rank p=0·08). The reasons for no R0 resection are reported in table 2.

The following analyses are based on the 711 patients who had curative resections. Table 3 shows the results of univariate analyses of 15-year overall survival and hazard ratios (HR), according to subgroups based on several prognostic variables. Overall survival was significantly higher for female patients who had D2 versus D1 dissection (35% *vs* 21%, p=0·03), and for patients with TNM stage II disease (33% in D2 *vs* 15% in D1, p=0·03). Patients with N2 disease had better survival after a D2 dissection (19% in D2 *vs* 0% in D1, p=0·07), as did those who had partial gastrectomy (35% in D2 *vs* 24% in D1, p=0·08), however this was not statistically significant.

Cause of death was analysed for all patients and the distribution for the D1 and D2 groups is summarised in table 4. Gastric-cancer-related death was significantly higher in the D1 group compared with the D2 group (HR 0·74 for D2 *vs* D1, 95% CI 0·59–0·93, p=0·01), whereas death due to other causes was not different between groups (HR 1·22 for D2 *vs* D1, 0·95–1·58, p=0·12; figure 4). Five patients in the D1 group had recurrence at the time of death (two patients with locoregional recurrence, two patients with local and distant recurrence,

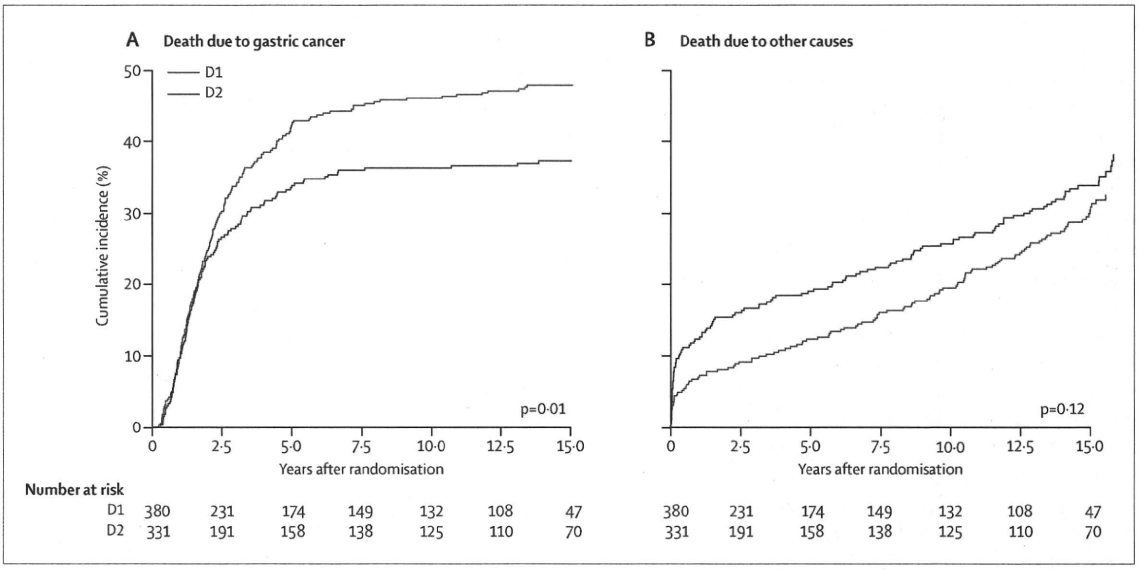

Figure 4: **Cumulative risk of death due to gastric cancer and due to other causes in patients treated with curative intent (n=711)**
D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy.

	D1 group (n=380)	D2 group (n=330)*
Alive, no recurrence†	82 (22%)	91 (28%)
Dead, no recurrence†	110 (29%)	107 (32%)
Dead, with recurrence‡	188 (49%)	131 (40%)
Local	82	40
Regional	73	43
Adjacent organs	37	26
Liver metastasis	65	37
Lung metastasis	12	6
Other organs	48	38
Dead, local+regional†	58 (15%)	42 (13%)
Dead, local+distant†	100 (26%)	56 (17%)
Dead, distant†	30 (8%)	34 (10%)

Data are number of patients (%). D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. *One patient is alive with recurrence. †Overall p value for recurrence pattern is 0·015. ‡Patients may be included in more than one category.

Table 5: **Recurrence sites and patterns at time of death**

and one patient with distant recurrence) and seven patients in the D2 group (three patients with locoregional recurrence, three patients with local and distant recurrence, and one patient with distant recurrence), although they died of other causes. In the group of patients who died from gastric cancer, postmortem examinations were done for ten patients in the D1 group and in 18 patients in the D2 group. Except for 31 patients in the D1 group and 25 patients in the D2 group, all recurrences were established by means of additional investigations (CT scan or ultrasonography, or both, biopsy or cytology, chest radiography or bonescan) depending on the recurrence.

Local recurrence was significantly higher in the D1 versus D2 group (82 of 380 [22%] *vs* 40 of 330 [12%]). Regional recurrence (73 of 380 [19%] in D1 *vs* 43 of 330 [13%] in D2) and liver metastases (65 of 380 [17%] in D1 *vs* 37 of 330 [11%] in D2) were also more common in the D1 group (table 5). Locoregional, distant, and distant-only recurrence are reported in table 5. Results for disease-free survival (p=0·31) and risk of recurrence (p=0·10) are shown in figure 5. Most patients had recurrence within 2–2·5 years, and after 5 years recurrence occurs less frequently. The difference in recurrence rates between the D1 and D2 group seems to present between 2·5 and 5 years of follow-up.

Patients older than 70 years had significantly lower overall survival in both the D1 and the D2 treatment groups, male patients had significantly lower survival compared with female patients in the D2 group (mean 7·07 years *vs* 8·73 years, p<0·0001), and patients undergoing splenectomy and pancreatectomy had significantly lower overall survival in both D1 and D2 (table 6). Subgroup analysis of patients without pancreatico-splenectomy (339 patients in D1 and 206 in D2, data not shown) shows a significantly higher overall survival in those who had D2 lymphadenectomy, with 15-year survival of 22% (75 of 339, 95% CI 17–26) in D1 and 35% (71 of 206, 29–42) in D2 (HR 1·34, 95% CI 1·09–1·65; log-rank p=0·006).

Discussion

Our findings based on 15-year follow-up data of the DGCT show that D2 lymphadenectomy is associated with lower locoregional recurrence and fewer gastric-cancer-related deaths than D1. The drawback of a D2 resection is its association with significantly higher

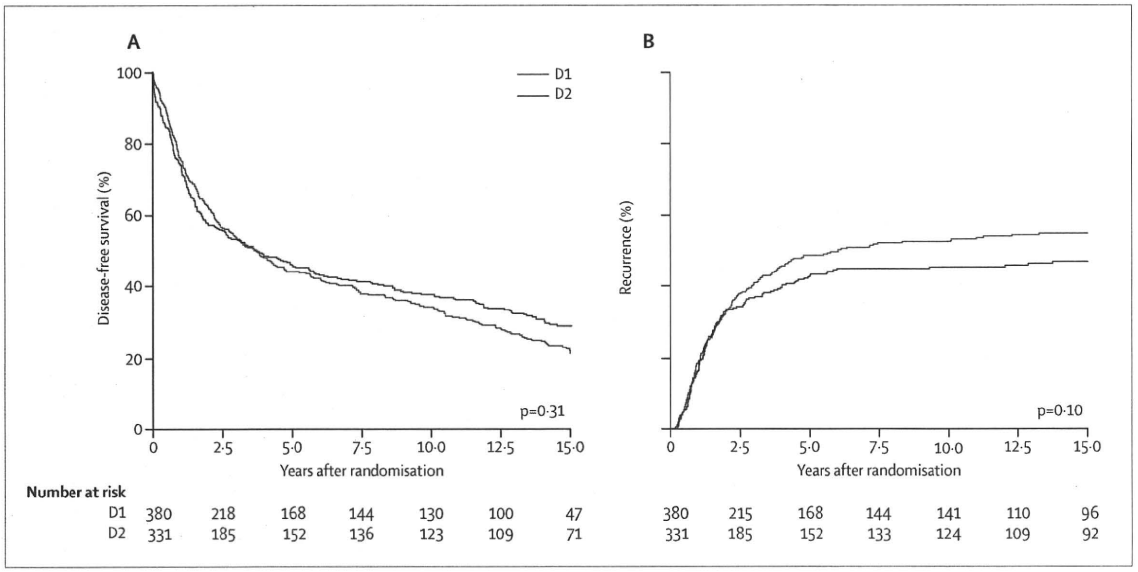

Figure 5: **Disease-free survival and risk of recurrence in patients treated with curative intent (n=711)**
D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy.

	N	D1 group mean OS (95% CI)	D2 group mean OS (95% CI)
Age			
≤70 years	481	8·23 (7·38–9·09)	8·69 (7·71–9·66)
>70 years	230	4·97 (4·10–5·82)	5·35 (4·21–6·49)
p value*	..	<0·0001	<0·0001
Sex			
Male	401	7·19 (6·33–8·05)	6·75 (5·75–7·74)
Female	310	7·07 (6·06–8·08)	8·73 (7·53–9·93)
p value*	..	0·83	0·02
Splenectomy			
Yes	165	5·14 (3·16–7·12)	5·19 (4·07–6·31)
No	546	7·37 (6·68–8·06)	9·09 (8·09–10·08)
p value*	..	0·02	<0·0001
Pancreatectomy			
Yes	108	2·34 (0·00–5·23)	4·85 (3·64–6·07)
No	603	7·27 (6·61–7·93)	8·81 (7·87–9·73)
p value*	..	0·0007	<0·0001

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. OS=overall survival. *Log-rank p value.

Table 6: **Effect of age, sex, splenectomy, and pancreatectomy on mean overall survival**

postoperative mortality and morbidity. However, at the time of the trial, resection of the spleen and pancreatic tail were regarded as necessary for adequate removal of D2 lymph-node stations 10 and 11 in proximal tumours, and in D1 in case of tumour invasion. Considering that a safer, spleen-preserving D2 resection is currently available in high-volume centres, and our findings of better recurrence and gastric-cancer-related survival rates, D2 resection now seems likely to be the recommended surgical approach for patients with resectable (curable) gastric cancer, despite the earlier follow-up results.

The extent of lymphadenectomy for curative resections in patients with gastric cancer has been debated for several decades. Better survival results reported for extended lymphadenectomy in Asian countries and better results with D2 in historical controls in western Europe and the USA were the reasons for the DGCT investigation. The MRC trial[2] in the UK addressed the same issue—eg, whether standardised D2 leads to better survival than D1 in patients with resectable gastric cancer. In both the MRC trial and the DGCT, no adjuvant chemotherapy was given. Both trials showed similar results: significantly increased morbidity and postoperative mortality after D2 surgery, without improvement in 5-year overall survival.[9,10] Long-term follow-up analysis of the DGCT after 11 years also showed no overall survival benefit with D2 lymphadenectomy.[1] In subgroup analysis, patients with N2 disease in the D2 group had higher survival than those in the D1 group (19% *vs* 0%, p=0·07). Since N2 disease is difficult to identify preoperatively, the conclusion was that extended lymph-node dissections might be beneficial if morbidity and mortality can be avoided. Meanwhile, it was reported that D2 lymphadenectomy can also be done safely in western Europe and the USA, without increased morbidity and mortality and after an adequate learning period, when routine pancreatico-splenectomy is avoided.[3,14]

After a median follow-up of 15 years after randomisation, these data show a more favourable recurrence pattern and a cancer-related survival benefit for patients who had a D2 rather than D1 lymphadenectomy. Patients older than 65 years, male sex and type of gastrectomy, splenectomy, and pancreatectomy were identified as important risk factors for the increased morbidity seen in the D2 group.[15] The increased mortality in this group of the pancreatico-splenectomy probably offset the survival benefit of D2 compared with D1 lymphadenectomy in the earlier analysis of the DGCT.

Current practice for treatment of patients with gastric cancer in Europe has become surgery with perioperative chemotherapy after the promising results of the MAGIC trial.[7] However, in the MAGIC trial, both D1 and D2 resections were done according to the discretion of the physician and 68% of the gastrectomies were D2 resections. A French trial[16] provided additional evidence for improved disease-free survival (DFS) and overall survival in resectable adenocarcinoma of the stomach and lower oesophagus with preoperative chemotherapy. Continuous infusion of fluorouracil and cisplatin resulted in a significantly higher R0 resection rate

	Time period	Surgery only			Multimodality treatment		
		N	RFS	OS	N	RFS	OS
MacDonald et al (2001)[6]	1991–1998	275	31% (3-year)	41% (3-year)	281 CRT	48% (3-year)	50% (3-year)
Cunningham et al (2006)[7]	1994–2002	253	NA	23% (5-year)	250 ECF	NA	36% (5-year)
Sakuramoto et al (2007)[8]	2001–2004	530	60% (3-year)	70% (3-year)	529 S-1	72% (3-year)	80% (3-year)
Boige et al (2007)[16]	1995–2003	111	21% (5-year)	24% (5-year)	113 FP	34% (5-year)	38% (5-year)

RFS=relapse-free survival. OS=overall survival. NA=not available. CRT=postoperative chemoradiotherapy (fluorouracil plus leucovorin followed by 45 Gy radiotherapy). ECF=Three preoperative and three postoperative cycles of epirubicin, cisplatin, and fluorouracil. S-1=cycles of S-1 (orally active combination of tegafur, gimeracil, and oteracil) for 1 year postoperatively. FP=2–3 cycles of preoperative fluorouracil and cisplatin; postoperative FP was recommended for patients with a response or stable disease with pN+.

Table 7: **Randomised trials of surgery only versus surgery combined with chemotherapy or chemoradiotherapy**

	Time period	Group 1				Group 2			
		N	Morbidity	Mortality	5-year overall survival	N	Morbidity	Mortality	5-year overall survival
Cuschieri et al (1999)[2]	1987–1994	200 (D1)	28%	6·5%	35%	200 (D2)	46%	13%	33%
Bonenkamp et al (1999)[9] and Hartgrink et al (2004)[1]	1989–1993	380 (D1)	25%	4%	45% and 30% (11-year)	331 (D2)	43%	10%	47% and 35% (11-year)
Degiuli et al (2004)[14]	1999–2002	76 (D1)	10·5%	1·3%	NA	86 (D2)	16·3%	0%	NA
Wu et al (2006)[4]	1993–1999	110 (D1)	7·3%	0%	53·6%	111 (D3)	17·1%	0%	59·5
Sasako et al (2008)[5]	1995–2001	263 (D2)	20·9%	0·8%	69·2%	260 (D2+PAND)	28·1%	0·8%	70·3

D1=standardised limited lymphadenectomy. D2=standardised extended lymphadenectomy. NA=not available. D3=referred to as D3 in this trial, but the same as D2. PAND=para-aortic nodal dissection.

Table 8: **Randomised trials comparing the extent of lymphadenectomy**

(73% *vs* 84%, p=0·04), a 13% improvement in 5-year DFS (21% *vs* 34%, p=0·003), and a 14% improvement in overall survival (24% *vs* 38%, p=0·02).[16] The INT0116 trial[6] found a significant improvement in 3-year overall survival, from 41% to 50%, with postoperative chemoradiotherapy in patients with adenocarcinoma of the stomach or gastro-oesophageal junction; only 10% of the patients had D2 resections, most had gastrectomy with D0 or D1 lymph-node dissection. The significant improvement in survival seemed to be merely a compensation of inadequate surgery. Nevertheless, as a result of this trial, a combination of surgery and postoperative chemoradiotherapy became the standard treatment for curable gastric cancer in the USA. A randomised controlled trial in Japanese patients with gastric cancer showed a significant improvement in 3-year overall survival with postoperative adjuvant chemotherapy with S-1 after D2, from 70·1% to 80·1%, compared with D2 dissection alone[8] (table 7). In non-Asian patients, however, meta-analyses of adjuvant chemotherapy showed only a small advantage in survival, insufficient to be considered standard of care.[17–21]

The results of these studies suggest that D2 surgery alone results in much better survival than limited surgery plus adjuvant CRT, as shown by the INT0116 trial, and that (neo)adjuvant therapy improves results even after D2 resection. Promising results from the Maruyama Index[22–24] and nomograms[25–27] that predict disease-specific survival might also help distinguish between patients with a high risk of relapse, and select those who will most likely benefit from tailored multimodality treatment. Because there are no other studies with such long-term follow-up data, including the MRC trial with similar design, the results of the DGCT are unique and of paramount clinical relevance. The main outcomes of the surgical trials discussed in this paper are summarised in table 8.

In selecting patients with gastric cancer for surgery, we do not think that elderly patients should be denied surgery. However, we cannot advocate extensive surgery, especially in elderly male compared with female patients. In this study, patients treated without curative intent were excluded from the analyses, according to the study protocol, and analyses were by intention to treat. Postoperative deaths were excluded from the analyses in one example as an illustration of whether patients would benefit from a D2 resection if excessive postoperative mortality could be prevented. Nowadays, surgery for gastric cancer can be done with a spleen-preserving and pancreas-preserving D2 resection technique (unless removal is indicated because of tumour invasion into these organs).[15,28–30] This technique is safer and is applicable in experienced, high-volume hospitals, where the outcome of patients with gastric cancer has been shown to be better.[31–33] Several countries (eg, Scandinavian countries) have already implemented a high-volume hospital setting and centralisation for gastric-cancer treatment, because it is recognised as high-risk surgery. Bilimoria and colleagues[34] studied the effect of differences in hospital surgical volume on perioperative mortality and long-term survival using the National Cancer Data Base. When comparing low-volume hospitals with high-volume hospitals, hazard ratios for perioperative mortality were substantially larger than for long-term survival. The effect of hospital volume in the DGCT has also been analysed and published,[9] and no association was noted between the number of patients randomised at centres (five *vs* five–14 *vs* ≥15 patients per centre) and complication, hospital death, or reoperation rates (webappendix pp 2). However, the importance of standardisation and surgical training is shown in a population-based study from the Netherlands.[35] The 5-year overall and relative survival of patients with curatively resected non-cardia gastric cancer was evaluated over time on a regional basis. The evaluation periods were before, during, and after the DGCT. Survival improved over time, most likely because of the standardisation and surgical training in D1 and D2 resection during the national DGCT, with extensive quality control measures for surgery and pathology implemented and monitored. With 5-year survival of 45% and 47% and 11-year survival of 30% and 35% for D1 and D2, respectively,[1] the results are the best recorded survival rates in western Europe and the USA.

Our results suggest that a D2 resection provides better locoregional control and significantly better cancer-specific survival compared with limited D1 surgery. Still, the significantly better gastric-cancer-specific survival

after D2 has to be interpreted carefully; a p value of <0·01 could be considered appropriate, since not all patients had post-mortem examinations (this was not mandatory according to the trial protocol) to confirm death due to gastric cancer.

Since a D2 resection can now be done safely with the spleen-preserving method, and more extended resections (D2 plus para-aortic nodal dissection) do not further improve survival outcome,[5] we believe D2 resection should be recommended as the standard surgical approach to resectable gastric cancer. With the surgical standard determined, a logical next step for investigation is whether patients with low Maruyama Index derive a survival benefit and improved locoregional control from chemoradiotherapy combined with gastrectomy and optimum standardised lymphadenectomy—ie, D2 resection without splenectomy—since treatment of gastric cancer requires a multidisciplinary approach. These issues are currently being addressed in the CRITICS trial (participating countries: the Netherlands, Sweden, and Denmark), a multicentre randomised phase 3 trial investigating whether chemoradiotherapy (45 Gy in 5 weeks with daily cisplatin and capecitabine) after preoperative chemotherapy (3×ECC [epirubicin, cisplatin, capecitabine]) and standardised D2 surgery without splenectomy leads to improved survival compared with postoperative chemotherapy (3×ECC).

For more on the **CRITICS trial** see http://www.critics.nl

Contributors

CJHV conceived and designed the trial. MS instructed surgeons during the first 6 months of the trial. Data collection was done by EM-KK. HP and IS did the statistical analyses. IS wrote the report with revisions from all other authors.

Conflicts of interest

The authors declared no conflicts of interest.

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References

1 Hartgrink HH, van de Velde CJ, Putter H, et al. Extended lymph-node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. *J Clin Oncol* 2004; **22:** 2069–77.

2 Cuschieri A, Weeden S, Fielding J, et al. Patient survival after D1 and D2 resections for gastric cancer: long-term results of the MRC randomized surgical trial. Surgical Co-operative Group. *Br J Cancer* 1999; **79:** 1522–30.

3 Degiuli M, Sasako M, Ponti A, Calvo F. Survival results of a multicentre phase II study to evaluate D2 gastrectomy for gastric cancer. *Br J Cancer* 2004; **90:** 1727–32.

4 Wu CW, Hsiung CA, Lo SS, et al. Nodal dissection for patients with gastric cancer: a randomised controlled trial. *Lancet Oncol* 2006; **7:** 309–15.

5 Sasako M, Sano T, Yamamoto S, et al. D2 lymphadenectomy alone or with para-aortic nodal dissection for gastric cancer. *New Engl J Med* 2008; **359:** 453–62.

6 Macdonald JS, Smalley SR, Benedetti J, et al. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345:** 725–30.

7 Cunningham D, Allum WH, Stenning SP, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355:** 11–20.

8 Sakuramoto S, Sasako M, Yamaguchi T, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007; **357:** 1810–20.

9 Bonenkamp JJ, Songun I, Hermans J, et al. Randomised comparison of morbidity after D1 and D2 dissection for gastric cancer in 996 Dutch patients. *Lancet* 1995; **345:** 745–48.

10 Bonenkamp JJ, Sasako M, Hermans J, et al. Extended lymph-node dissection for gastric cancer. *N Engl J Med* 1999; **340:** 908–14.

11 Putter H, Sasako M, Hartgrink HH, van de Velde CJH, van Houwelingen JC. Long-term survival with non-proportional hazards: results from the Dutch Gastric Cancer Trial. *Statist Med* 2005; **24:** 2807–21.

12 Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. *Statist Med* 2007; **26:** 2389–430.

13 Fine J, Gray R. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc* 1999; **94:** 496–509.

14 Degiuli M, Sasako M, Calgaro M, et al. Morbidity and mortality after D1 and D2 gastrectomy for cancer: interim analysis of the Italian Gastric Cancer Study Group (IGCSG) randomised surgical trial. *Eur J Surg Oncol* 2004; **30:** 303–08.

15 Sasako M, Dutch Gastric Cancer Study Group. Risk factors for surgical treatment in the Dutch gastric cancer trial. *Br J Surg* 1997; **84:** 1567–71.

16 Boige V, Pignon J, Saint-Aubert B, et al. Final results of a randomized trial comparing preoperative 5-fluorouracil (F)/cisplatin (P) to surgery alone in adenocarcinoma of stomach and lower esophagus (ASLE): FNLCC ACCORD07-FFCD 9703 trial. *J Clin Oncol* 2007; **25** (18 suppl): 200S.

17 Earle CC, Maroun JA. Adjuvant chemotherapy after curative resection for gastric cancer in non-Asian patients: revisiting a meta-analysis of randomised trials. *Eur J Cancer* 1999; **35:** 1059–64.

18 Gianni L, Panzini I, Tassinari D, Mianulli AM, Desiderio F, Ravaioli A. Meta-analyses of randomized trials of adjuvant chemotherapy in gastric cancer. *Ann Oncol* 2001; **12:** 1178–80.

19 Hermans J, Bonenkamp JJ, Boon MC, et al. Adjuvant therapy after curative resection for gastric cancer: meta-analysis of randomized trials. *J Clin Oncol* 1993; **11:** 1441–47.

20 Mari E, Floriani I, Tinazzi A, et al. Efficacy of adjuvant chemotherapy after curative resection for gastric cancer: a meta-analysis of published randomised trials. A study of the GISCAD (Gruppo Italiano per lo Studio dei Carcinomi dell'Apparato Digerente). *Ann Oncol* 2000; **11:** 837–43.

21 Wagner AD, Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. *J Clin Oncol* 2006; **24:** 2903–09.

22 Peeters KC, Hundahl SA, Kranenbarg EK, Hartgrink H, van de Velde CJ. Low Maruyama index surgery for gastric cancer: blinded reanalysis of the Dutch D1-D2 trial. *World J Surg* 2005; **29:** 1576–84.

23 Hundahl SA, Peeters KC, Kranenbarg EK, Hartgrink H, van de Velde CJ. Improved regional control and survival with "low Maruyama Index" surgery in gastric cancer: autopsy findings from the Dutch D1-D2 Trial. *Gastric Cancer* 2007; **10:** 84–86.

24 Hundahl SA, Macdonald JS, Benedetti J, Fitzsimmons T, Southwest Oncology Group and the Gastric Intergroup. Surgical treatment variation in a prospective, randomized trial of chemoradiotherapy in gastric cancer: the effect of undertreatment. *Ann Surg Oncol* 2002; **9:** 278–86.

25 Kattan MW, Karpeh MS, Mazumdar M, Brennan MF. Postoperative nomogram for disease-specific survival after an R0 resection for gastric carcinoma. *J Clin Oncol* 2003; **21:** 3647–50.

26 Peeters KC, Kattan MW, Hartgrink HH, et al. Validation of a nomogram for predicting disease-specific survival after an R0 resection for gastric carcinoma. *Cancer* 2005; **103:** 702–07.

27 Novotny AR, Schuhmacher C, Busch R, Kattan MW, Brennan MF, Siewert JR. Predicting individual survival after gastric cancer resection: validation of a US-derived nomogram at a single high-volume center in Europe. *Ann Surg* 2006; **243:** 74–81.

28 Csendes A, Burdiles P, Rojas J, Braghetto I, Diaz JC, Maluenda F. A prospective randomized study comparing D2 total gastrectomy versus D2 total gastrectomy plus splenectomy in 187 patients with gastric carcinoma. *Surgery* 2002; **131:** 401–07.

29 Yu W, Choi GS, Chung HY. Randomized clinical trial of splenectomy versus splenic preservation in patients with proximal gastric cancer. *Br J Surg* 2006; **93**: 559–63.

30 Wang JY, Huang TJ, Chen FM, Huang CJ, Huang YS, Hsieh JS. A comparative study of pancreatectomy and pancreas-preserving gastrectomy in advanced gastric carcinomas. *Hepatogastroenterology* 2004; **51**: 1229–32.

31 Meyer HJ. The influence of case load and the extent of resection on the quality of treatment outcome in gastric cancer. *Eur J Surg Oncol* 2005; **31**: 595–04.

32 Bachmann MO, Alderson D, Edwards D, et al. Cohort study in South and West England of the influence of specialization on the management and outcome of patients with oesophageal and gastric cancers. *Br J Surg* 2002; **89**: 914–22.

33 Birkmeyer JD, Stukel TA, Siewers AE, Goodney PP, Wennberg DE, Lucas FL. Surgeon volume and operative mortality in the United States. *N Engl J Med* 2003; **349**: 2117–27.

34 Bilimoria KY, Bentrem DJ, Feinglass JM, et al. Directing surgical quality improvement initiatives: comparison of perioperative mortality and long-term survival for cancer surgery. *J Clin Oncol* 2008; **26**: 4626–33.

35 Krijnen P, den Dulk M, Meershoek-Klein Kranenbarg E, Jansen-Landheer MLEA, van de Velde CJH. Improved survival after resectable non-cardia gastric cancer in the Netherlands: the importance of surgical training and quality control. *Eur J Surg Oncol* 2009; **35**: 715–20.

Benefit of Adjuvant Chemotherapy for Resectable Gastric Cancer

A Meta-analysis

The GASTRIC (Global Advanced/Adjuvant Stomach Tumor Research International Collaboration) Group*

Context Despite potentially curative resection of stomach cancer, 50% to 90% of patients die of disease relapse. Numerous randomized clinical trials (RCTs) have compared surgery alone with adjuvant chemotherapy, but definitive evidence is lacking.

Objectives To perform an individual patient-level meta-analysis of all RCTs to quantify the potential benefit of chemotherapy after complete resection over surgery alone in terms of overall survival and disease-free survival, and to further study the role of regimens, including monochemotherapy; combined chemotherapy with fluorouracil derivatives, mitomycin C, and other therapies but no anthracyclines; combined chemotherapy with fluorouracil derivatives, mitomycin C, and anthracyclines; and other treatments.

Data Sources Data from all RCTs comparing adjuvant chemotherapy with surgery alone in patients with resectable gastric cancer. We searched MEDLINE (up to 2009), the Cochrane Central Register of Controlled Trials, the National Institutes of Health trial registry, and published proceedings from major oncologic and gastrointestinal cancer meetings.

Study Selection All RCTs closed to patient recruitment before 2004 were eligible. Trials testing radiotherapy; neoadjuvant, perioperative, or intraperitoneal chemotherapy; or immunotherapy were excluded. Thirty-one eligible trials (6390 patients) were identified.

Data Extraction As of 2010, individual patient data were available from 17 trials (3838 patients representing 60% of the targeted data) with a median follow-up exceeding 7 years.

Results There were 1000 deaths among 1924 patients assigned to chemotherapy groups and 1067 deaths among 1857 patients assigned to surgery-only groups. Adjuvant chemotherapy was associated with a statistically significant benefit in terms of overall survival (hazard ratio [HR], 0.82; 95% confidence interval [CI], 0.76-0.90; $P<.001$) and disease-free survival (HR, 0.82; 95% CI, 0.75-0.90; $P<.001$). There was no significant heterogeneity for overall survival across RCTs ($P=.52$) or the 4 regimen groups ($P=.13$). Five-year overall survival increased from 49.6% to 55.3% with chemotherapy.

Conclusion Among the RCTs included, postoperative adjuvant chemotherapy based on fluorouracil regimens was associated with reduced risk of death in gastric cancer compared with surgery alone.

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ALTHOUGH EPIDEMIOLOGICAL studies describe a reduction in recent years in gastric cancer incidence, gastric cancer is a common and highly fatal disease, with current 5-year survival rates less than 20%.[1] Surgery for disease at an early stage can usually be performed with curative intent, but the 5-year survival rate is disappointing.[2,3] Over the last 3 decades, numerous phase 3 studies including a surgery-only group have been reported, but definitive evidence of the efficacy of adjuvant chemotherapy is lacking. Recently, the large-scale Japanese phase 3 trial by the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) group[4] reported the superiority of S-1 as an adjuvant chemotherapy over surgery alone after D2 lymph node dissection. Its applicability outside of East Asia is uncertain, and the First-Line Advanced Gastric Cancer Study (FLAGS) in advanced disease[5] that compared cisplatin and S-1 vs cisplatin and fluoropyridines in non-Asian countries was negative. Therefore, standard management following curative surgery is heterogeneous throughout the world.

See also pp 1723, 1753 and Patient Page.

No patient-level meta-analyses have been carried out to date. Based on published results, recent meta-analyses[6-10] indicated that adjuvant chemotherapy produces a small survival benefit, if any, in patients with resected gastric carcinoma (eTable 1, available at http://www.jama.com) but did not recommend adjuvant chemotherapy as routine therapy. Since then, several additional trials have been conducted in this setting. Overall,

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