

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: McClung MR, Lewiecki EM, Cohen SB, et al. Denosumab in postmenopausal women with low bone mineral density. *N Engl J Med* 2006;354:821-31.

Online Appendix – Detailed Description of Antibody Assay Methods

The electrochemiluminescent (ECL) bridging assay was performed using an M-Series M8 analyzer (BioVeris Corporation, Gathersburg, MD). A mixture of biotinylated and ruthenylated denosumab was incubated overnight with duplicate undiluted serum samples. Next, paramagnetic streptavidin beads were added, and a magnetic field was used to isolate bead-antibody complexes. An ECL signal proportional to the quantity of bound anti-denosumab antibodies was produced by the addition of tripropylamine, which reacted with the bound ruthenium when a voltage was applied. The negative control was pooled human serum, and the positive control was an affinity-purified rabbit polyclonal anti-denosumab antibody. The ratio of a sample's mean ECL value to that of the negative control (n=8 replicates) was defined as the signal-to-noise ratio. Post-dose samples were considered positive for antibody if the signal-to-noise ratio was at least 1.2 and the mean ECL value was at least 2-fold greater than the corresponding pre-dose sample. Pre-dose samples were considered positive for antibody if the signal-to-noise ratio was at least 1.2 and the mean ECL value was at least 2-fold greater than the negative control. The assay sensitivity was 10 ng/mL. Intraassay and interassay coefficients of variation were both less than 6% at the limit of detection.

Samples with a signal-to-noise ratio of at least 1.2 in the ECL assay were tested for neutralizing activity in a cell-based bioassay. In response to RANKL, RAW264.7 cells differentiate into osteoclast-like cells and produce tartrate-resistant acid phosphatase (TRAP) mRNA, which is blocked by denosumab. Diluted (1:20) triplicate samples were tested simultaneously in neutralizing antibody screening and specificity assays. Samples were incubated with denosumab (10 ng/mL) and RANKL (2 ng/mL) and then added to RAW264.7 cells (screening assay), or incubated with RAW264.7 cells alone (specificity assay). After incubation for 48 ± 4 hours, cells were lysed and TRAP mRNA was detected by a sandwich assay, using Branched DNA Technology. The assay yielded a luminescent signal measured in counts per second (CPS). Samples and controls were tested in triplicate. In both the screening and the specificity bioassays, the negative control was pooled human serum. In the screening bioassay, the positive

control was an affinity-purified, polyclonal, rabbit anti-denosumab antibody. A screening assay value was obtained for each sample by dividing the mean CPS value by the negative control value. Post-dose samples were considered positive for neutralizing antibodies if the screening assay value was at least 1.3 in the screening bioassay, the mean CPS value was at least 2-fold greater than the corresponding pre-dose sample, and the mean CPS value from the screening bioassay was at least 2-fold greater than that in the specificity bioassay. Pre-dose samples were considered positive for neutralizing antibodies if the first and third criteria above were met. The assay reliably detects at least 500 ng/mL of anti-denosumab in undiluted serum.

Ref: Moxness M, Tatarewicz S, Weeraratne D, Murakami N, Wullner D, Mytych D, Jawa V, Koren E, Swanson S. Immunogenicity Testing for Antibodies Directed Against Therapeutic Human Monoclonal Antibodies Using Electrochemiluminescent Detection. Clin Chem 2005;51:1983

Table A-1: Percentage Change From Baseline in Bone Mineral Density, Mean (SE)*

	Denosumab 3-monthly				Denosumab 6-monthly				
	Placebo (n = 46)	6 mg (n = 40)	14 mg (n = 43)	30 mg (n = 40)	14 mg (n = 53)	60 mg (n = 46)	100 mg (n = 41)	210 mg (n = 46)	ALN (n = 46)
Lumbar spine, 1 month, n	43	37	40	38	49	44	41	43	45
Mean (SE)	-0.2 (0.4)	1.2 (0.5)	1.8 (0.4) ^b	2.4 (0.5) ^{c,d}	1.2 (0.4)	1.6 (0.4) ^a	0.9 (0.4)	1.2 (0.4)	1.0 (0.4)
Lumbar spine, 12 months, n	40	36	35	32	48	41	37	41	45
Mean (SE)	-0.8 (0.5)	4.4 (0.5) ^{d,c}	4.7 (0.5) ^{d,c}	6.7 (0.5) ^{c,e}	3.0 (0.4) ^{c,d}	4.6 (0.5) ^c	5.5 (0.5) ^c	5.1 (0.5) ^c	4.6 (0.5) ^c
Total hip, 1 month, n	43	37	42	38	49	45	41	44	45
Mean (SE)	-0.1 (0.3)	1.1 (0.3) ^{b,d}	0.8 (0.3) ^a	0.9 (0.3) ^a	0.6 (0.2)	1.2 (0.3) ^{c,d}	0.5 (0.3)	0.5 (0.3)	0.4 (0.3)
Total hip, 12 months, n	40	36	36	32	48	42	37	41	45
Mean (SE)	-0.6 (0.4)	2.9 (0.4) ^c	2.5 (0.4) ^c	3.3 (0.4) ^{c,d}	1.9 (0.3) ^c	3.6 (0.4) ^{c,e}	2.5 (0.4) ^c	2.3 (0.4) ^c	2.1 (0.3) ^c
Femoral neck,	43	37	42	38	49	45	41	44	45

1 month, n									
Mean (SE)	0.0 (0.5)	1.8 (0.5) ^d	0.3 (0.5)	0.3 (0.5)	1.2 (0.4)	0.6 (0.5)	0.3 (0.5)	1.2 (0.5)	0.2 (0.5)
Femoral neck,	40	36	36	32	48	42	37	41	45
12 months, n									
Mean (SE)	-0.3 (0.5)	3.4 (0.5) ^c	2.1 (0.5) ^c	3.1 (0.6) ^c	2.1 (0.5) ^c	2.1 (0.5) ^c	2.3 (0.5) ^c	2.4 (0.5) ^c	2.1 (0.5) ^c
1/3 radius, 12	39	35	35	32	44	43	37	37	42
months, n									
Mean (SE)	-2.0 (0.5)	1.3 (0.6) ^{c,d}	0.4 (0.5) ^c	1.1 (0.6) ^{c,d}	0.9 (0.5) ^{c,d}	1.3 (0.5) ^{c,e}	1.1 (0.5) ^{c,d}	1.1 (0.5) ^{c,d}	-0.5 (0.5) ^a
Total body†,	36	36	35	28	43	41	34	38	39
12 months, n									
Mean (SE)	-0.2 (0.5)	1.8 (0.5) ^b	1.8 (0.5) ^b	2.8 (0.5) ^{dc}	0.6 (0.4)	2.4 (0.4) ^c	1.8 (0.5) ^b	2.1 (0.4) ^b	1.5 (0.4) ^b

ALN, alendronate.

*Both Least Squares Means and Standard Error are estimated from ANCOVA adjusted by geographic region, treatment group, and baseline value.

†Total body minus head BMD.

Comparison to placebo: ^aP < 0.05, ^bP < 0.01, ^cP < 0.001, adjusted by Hochberg's method for the multiple comparisons vs. placebo; nominal P-value is reported for alendronate vs placebo.

Denosumab comparison to alendronate: ^dP < 0.05, ^eP < 0.01, nominal P-value is reported.

Table A-2. Percentage Change From Baseline in Serum C-Telopeptide

	Denosumab 3-monthly				Denosumab 6-monthly				ALN (n = 46)
	Placebo (n = 46)	6 mg (n = 43)	14 mg (n = 44)	30 mg (n = 40)	14 mg (n = 53)	60 mg (n = 47)	100 mg (n = 41)	210 mg (n = 46)	
3 Days, n	45	42	44	40	52	47	41	46	44
Mean	-0.7	-68.4 ^{a,d}	-74.7 ^{a,d}	-79.2 ^{a,d}	-76.2 ^{a,d}	-81.7 ^{a,d}	-80.3 ^{a,d}	-82.9 ^{a,d}	-23.4 ^a
SE	2.4	2.5	2.5	2.6	2.2	2.4	2.5	2.4	2.4
Median	-1.9	-71.8	-77.6	-81.2	-80.1	-83.6	-81.5	-82.9	-20.9
1 Month, n	45	38	42	39	51	44	41	44	46
Mean	-4.2	-84.7 ^{a,d}	-86.5 ^{a,d}	-86.6 ^{a,d}	-87.0 ^{a,d}	-87.8 ^{a,d}	-87.0 ^{a,d}	-87.8 ^{a,d}	-61.9 ^a
SE	2.0	2.1	2.1	2.2	1.8	2.0	2.1	2.0	2.0
Median	-8.6	-85.9	-89.0	-89.5	-89.2	-89.4	-87.4	-89.2	-66.2
3 Months, n	42	37	39	37	52	45	40	46	46
Mean	-2.2	-57.0 ^a	-78.1 ^{a,c}	-79.4 ^{a,d}	-76.4 ^{a,c}	-86.0 ^{a,d}	-84.7 ^{a,d}	-87.0 ^{a,d}	-64.6 ^a
SE	3.0	3.2	3.1	3.2	2.7	2.9	3.0	2.9	2.9
Median	-9.5	-60.8	-80.6	-86.8	-83.1	-88.2	-86.5	-88.7	-68.4
6 Months, n	38	34	33	33	49	42	37	42	34
Mean	-0.6	-65.3 ^a	-78.0 ^{a,b}	-77.2 ^{a,b}	-34.8 ^{a,d}	-70.1 ^a	-72.7 ^a	-83.1 ^{a,d}	-64.5 ^a
SE	3.8	3.9	4.0	4.1	3.3	3.6	3.8	3.6	3.9

Median	-2.0	-66.6	-85.6	-83.7	-39.8	-72.4	-82.9	-87.6	-68.7
6 Months +3 Days, n	35	32	31	30	46	41	32	40	34
Mean	6.7	-78.8 ^{a,b}	-84.7 ^{a,d}	-81.3 ^{a,c}	-77.0 ^a	-83.8 ^{a,d}	-83.5 ^{a,c}	-85.6 ^{a,d}	-70.1 ^a
SE	2.9	3.0	3.1	3.2	2.5	2.7	3.0	2.7	2.9
Median	2.2	-82.9	-87.5	-87.8	-80.0	-84.6	-85.5	-88.8	-72.7
9 Months, n	39	37	38	32	48	44	38	42	44
Mean	7.0	-63.9 ^a	-74.9 ^{a,b}	-79.7 ^{a,c}	-74.9 ^{a,b}	-85.3 ^{a,d}	-82.0 ^{a,d}	-85.2 ^{a,d}	-66.0 ^a
SE	3.0	3.0	3.0	3.3	2.7	2.8	3.0	2.9	2.8
Median	-0.9	-68.1	-81.1	-85.4	-82.2	-87.0	-85.9	-87.8	-70.8
12 Months, n	36	35	36	31	47	41	36	42	40
Mean	-0.3	-55.9 ^a	-60.7 ^a	-71.4 ^a	-14.1 ^d	-62.9 ^a	-70.9 ^a	-72.3 ^a	-66.8 ^a
SE	5.7	5.7	5.7	6.2	4.9	5.4	5.6	5.2	5.4
Median	-4.7	-61.4	-78.0	-87.2	-12.1	-70.8	-78.7	-84.1	-72.6

ALN, alendronate

*Least Squares Means and Standard Error are estimated from ANCOVA adjusted by geographic region, treatment group, and baseline value.

Comparison to placebo: ^aP < 0.001, and P-value is adjusted by Hochberg's method for the multiple comparisons between each denosumab dose group vs placebo; nominal P-value is reported for alendronate vs placebo.

Denosumab comparison to alendronate: ^bP < 0.05, ^cP < 0.01, and ^dP < 0.001, nominal P-value is reported.

Table A-3. Percentage Change From Baseline in Urine N-Telopeptide/Creatinine

	Denosumab 3-Monthly				Denosumab 6-Monthly				ALN (n = 46)
	Placebo (n = 46)	6 mg (n = 43)	14 mg (n = 44)	30 mg (n = 40)	14 mg (n = 53)	60 mg (n = 47)	100 mg (n = 41)	210 mg (n = 46)	
3 Days, n	45	42	43	40	51	47	41	46	44
Mean	-3.6	-48.7 ^{a,d}	-55.1 ^{a,d}	-58.3 ^{a,d}	-53.1 ^{a,d}	-58.9 ^{a,d}	-59.7 ^{a,d}	-60.8 ^{a,d}	-13.3
SE	3.2	3.3	3.3	3.4	3.0	3.2	3.3	3.1	3.2
Median	-4.8	-48.3	-60.6	-60.6	-56.4	-63.2	-56.5	-65.1	-15.8
1 Month, n	44	38	41	39	50	44	41	43	46
Mean	0.3	-57.7 ^{a,c}	-62.8 ^{a,d}	-63.5 ^{a,d}	-59.8 ^{a,d}	-61.9 ^{a,d}	-64.2 ^{a,d}	-63.1 ^{a,d}	-41.1 ^a
SE	3.8	4.1	4.0	4.1	3.6	3.9	3.9	3.8	3.7
Median	-7.3	-61.8	-66.2	-68.4	-64.7	-65.9	-71.6	-71.5	-44.6
3 Months, n	41	37	39	37	52	45	40	46	46
Mean	4.3	-27.1 ^a	-52.2 ^{a,b}	-57.7 ^{a,d}	-50.6 ^{a,b}	-64.0 ^{a,d}	-60.5 ^{a,d}	-60.5 ^{a,d}	-38.1 ^a
SE	4.2	4.4	4.3	4.5	3.7	4.0	4.2	4.0	3.9
Median	-0.4	-34.7	-53.8	-63.1	-53.2	-62.8	-59.1	-67.0	-46.0
6 Months, n	37	34	33	33	49	43	37	42	34
Mean	3.8	-44.9 ^a	-48.4 ^a	-46.9 ^a	-21.2 ^{a,c}	-39.8 ^a	-46.7 ^a	-54.0 ^a	-43.9 ^a
SE	5.3	5.4	5.6	5.6	4.5	4.9	5.2	4.9	5.4

Median	-3.6	-47.9	-59.7	-65.6	-25.2	-51.6	-61.9	-63.4	-48.0
6 Months + 3 Days, n	35	31	29	30	44	41	32	40	34
Mean	-3.0	-48.7 ^a	-55.7 ^a	-57.9 ^a	-61.0 ^a	-55.8 ^a	-59.5 ^a	-62.6 ^a	-49.6 ^a
SE	5.1	5.4	5.7	5.6	4.6	4.8	5.4	4.8	5.2
Median	-10.0	-60.1	-61.2	-67.5	-66.9	-61.2	-69.0	-71.9	-55.0
9 Months, n	39	37	38	32	48	44	38	42	44
Mean	0.9	-44.0 ^a	-48.3 ^a	-54.6 ^a	-43.8 ^a	-61.7 ^{a,b}	-62.0 ^{a,b}	-59.0 ^a	-48.5 ^a
SE	4.8	4.8	4.8	5.3	4.2	4.5	4.8	4.5	4.4
Median	-3.4	-44.1	-60.9	-71.3	-50.7	-66.5	-69.9	-66.0	-54.8
12 Months, n	36	35	36	31	47	40	36	42	40
Mean	21.1	-31.8 ^a	-30.6 ^a	-50.8 ^{a,b}	5.5 ^d	-27.2 ^a	-36.7 ^a	-39.4 ^a	-33.0 ^a
SE	6.3	6.3	6.3	6.9	5.5	6.0	6.2	5.8	5.9
Median	24.3	-40.0	-54.7	-60.7	-1.2	-34.9	-51.3	-58.5	-45.4

ALN, alendronate

*Least Squares Means and Standard Error are estimated from ANCOVA adjusted by geographic region, treatment group, and baseline value.

Comparison to placebo: ^aP < 0.001, and P-value is adjusted by Hochberg's method for the multiple comparisons between each denosumab dose group vs placebo; nominal P-value is reported for alendronate vs placebo.

Denosumab comparison to alendronate: ^bP < 0.05, ^cP < 0.01, and ^dP < 0.001, nominal P-value is reported.

Table A-4. Percentage Change From Baseline in Bone-Specific Alkaline Phosphatase

	Denosumab 3-Monthly				Denosumab 6-Monthly				ALN (n = 46)
	Placebo (n = 46)	6 mg (n = 43)	14 mg (n = 44)	30 mg (n = 40)	14 mg (n = 53)	60 mg (n = 47)	100 mg (n = 41)	210 mg (n = 46)	
	1 Month, n	44	38	40	39	51	44	41	
Mean	16.5	1.3	-2.1	-2.9	-1.3	-3.7	-0.7	-9.9	-3.3
SE	6.9	7.3	7.2	7.4	6.4	6.9	7.1	6.8	6.7
Median	8.8	-12.1	-17.2	-13.9	-5.8	-11.4	-6.4	-11.5	-13.0
3 Months, n	41	37	39	36	50	45	39	45	45
Mean	14.0	-46.6 ^a	-50.9 ^a	-56.2 ^{a,b}	-49.5 ^a	-54.7 ^{a,b}	-49.7 ^a	-55.6 ^{a,b}	-39.9 ^a
SE	4.9	5.1	5.0	5.2	4.4	4.7	4.9	4.6	4.6
Median	-1.6	-54.5	-59.2 ^b	-63.4	-47.1	-61.2	-58.5 ^b	-59.1 ^b	-44.8
6 Months, n	38	33	32	32	48	42	35	41	34
Mean	-3.4	-53.9 ^a	-62.9 ^a	-64.1 ^a	-41.4 ^{a,c}	-56.1 ^a	-62.2 ^a	-60.7 ^a	-55.1 ^a
SE	3.6	3.8	3.9	4.0	3.2	3.5	3.7	3.5	3.8
Median	-0.2	-57.6	-67.3	-72.5	-46.6	-61.1	-64.1	-64.4	-59.4
9 Months, n	37	36	37	32	47	43	38	42	44
Mean	-5.0	-62.3 ^a	-65.0 ^a	-65.7 ^a	-61.5 ^a	-65.6 ^a	-69.2 ^a	-66.4 ^a	-60.6 ^a

SE	3.5	3.5	3.6	3.8	3.1	3.3	3.4	3.3	3.2
Median	-4.9	-62.9	-67.8	-74.8	-68.4	-73.3	-71.8	-71.5	-67.0
12 Months, n	27	31	30	24	30	33	26	34	32
Mean	-8.4	-63.4 ^a	-62.5 ^a	-65.8 ^a	-49.8 ^a	-62.5 ^a	-65.9 ^a	-59.1 ^a	-60.9 ^a
SE	4.6	4.2	4.3	4.9	4.2	4.2	4.6	4.0	4.2
Median	-19.2	-62.7	-64.2	-72.6	-50.2	-68.3	-68.6	-68.3	-62.8

ALN, alendronate.

*Least Squares Means and Standard Error are estimated from ANCOVA adjusted by geographic region, treatment group, and baseline value.

Comparison to placebo: ^aP < 0.001, and P-value is adjusted by Hochberg's method for the multiple comparisons between each denosumab dose group vs placebo; nominal P-value is reported for alendronate vs placebo.

Denosumab comparison to alendronate: ^bP < 0.05, ^cP < 0.01, and ^dP < 0.001, nominal P-value is reported.